APPENDIX 1

PATENT

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SPECIFICATION

OF

BRUCE P. NERI, JOHN S. CURTIS,

MARK L. COLLINS AND DANAHEY RYAN

FOR

CLOSED VESSEL FOR ISOLATING TARGET MOLECULES AND FOR PERFORMING AMPLIFICATION

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This application is a continuation-in-part of U.S.S.N. 922,155, entitled TARGET AND BACKGROUND CAPTURE METHODS FOR AFFINITY ASSAYS, filed October 23, 1986 and U.S.S.N. 136,920, entitled TARGET/BACKGROUND CAPTURE WITH AMPLIFICATION, filed December 21, 1987 and hereby incorporated by reference.

The present invention pertains to methods reagents, compositions, kits, and instruments for use in capturing target molecules. One embodiment of the present invention features a substantially closed containment vessel for capturing deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) from clinical samples. Further embodiments of the present invention feature a closed containment vessel for detecting the formation of nucleic acid hybridization reactions and for amplifying signals related to such hybridization reactions. Embodiments of the present invention provide methods and articles of manufacture for rapid, sensitive detection of target molecules in clinical samples.

The following definitions are provided to facilitate an understanding of the present invention. The term "biological binding pair" as used in the present application refers to any pair of molecules which exhibit natural affinity or binding capacity. For the purposes of the present application, the term "ligand" will refer to one molecule of the biological binding pair and the term "antiligand" or "receptor" will refer to the opposite molecule of the biological binding pair. For example, without limitation, embodiments of the present invention have applications in nucleic acid hybridization assays where the biological binding pair includes two complementary strands of polynucleic acid. One of the strands

is designated the ligand and the other strand is designated the antiligand. However, the biological binding pair may include antigens and antibodies, proteins and protein binders, chelators, drugs, and drug receptor sites and enzymes and enzyme substrates.

The term "probe" refers to a ligand of known qualities capable of selectively binding to a target antiligand. As applied to nucleic acids, the term "probe" refers to a strand of nucleic acid having a base sequence complementary to a target strand.

The term "label" refers to a molecular moiety capable of detection including, by way of example, without limitation, radioactive isotopes, enzymes, luminescent agents, and dyes. The term "agent" is used in a broad sense, including any molecular moiety which participates in reactions which lead to a detectable response. The term "cofactor" is used broadly to include any molecular moiety which participates in reactions with the agent.

The term "retrievable" is used in a broad sense to describe an entity which can be substantially dispersed within a medium and removed or separated from the medium by immobilization, filtering, binding, partitioning, or the like.

The term "support" when used alone includes conventional supports such as filters and membranes as well as retrievable solid supports.

The term "reversible," in regard to the binding of ligands and antiligands, means capable of binding and releasing upon changes in the environment which do not permanently alter the gross chemical nature of the ligand and antiligand. For example, without limitation, reversible binding of nucleic acid ligands and antilands is controlled by changes in the pH, temperature, and ionic strength which do not destroy the ligand or antiligand.

The term "amplify" is used in the broad sense to mean creating an amplification product which may include by way or

example, additional target molecules, or target-like molecules which are capable of functioning in a manner like the target molecule, or a molecule subject to detection steps in place of the target molecule, which molecules are created by virtue of the presence of the target molecule in the sample. In the situation where the target is a polynucleotide, additional target, or target-like molecules, or molecules subject to detecting can be made enzymatically with DNA or RNA polymerases or transcriptases.

The terms "closed" and "contained" are used to suggest that reactions with respect to a sample do not share physical hardware, which reactants may come in contact, with any other sample, and such reactions are performed in an atmosphere unique to such sample and reactants.

Genetic information is stored in living cells in threadlike molecules of DNA. In vivo, the DNA molecule is a double helix, each strand of which is a chain of nucleotides. Each nucleotide is characterized by one of four bases: adenine (A), guanine (G), thymine (T), and cytosine (C). The bases are complementary in the sense that, due to the orientation of functional groups, certain base pairs attract and bond to each other through hydrogen bonding. Adenine in one strand of DNA pairs with thymine in an opposing complementary strand. Guanine in one strand of DNA pairs with cytosine in an opposing complementary strand. In RNA, the thymine base is replaced by uracil (U) which pairs with adenine in an opposing complementary strand.

DNA consists of covalently linked chains of deoxyribonucleotides. RNA consists of covalently linked chains of ribonucleotides. The genetic code of a living organism is carried upon DNA in the sequence of the base pairs. Living organisms use RNA to transcribe and translate the genetic code into proteins.

Each nucleic acid is linked by a phosphodiester bridge between the five prime hydroxyl group of the sugar of one nucleotide and the three prime hydroxyl group of the sugar of an adjacent nucleotide. Each linear strand of naturally occurring DNA or RNA has one terminal end having a free five prime hydroxyl group and another terminal end having a three prime hydroxyl group. The terminal ends of polynucleotides are often referred to as being five prime termini or three prime termini in reference to the respective free hydroxyl group. Complementary strands of DNA and RNA form antiparallel complexes in which the three prime terminal end of one strand is oriented to the five prime terminal end of the opposing strand.

Nucleic acid hybridization assays are based on the tendency of two nucleic acid strands to pair at complementary regions. Presently, nucleic acid hybridization assays are primarily used to detect and identify unique DNA or RNA base sequences or specific genes in a complete DNA molecule, in mixtures of nucleic acid, or in mixtures of nucleic acid fragments.

The identification of unique DNA or RNA sequences of specific genes within the total DNA or RNA extracted from tissue or culture samples may indicate the presence of physiological or pathological conditions. In particular, the identification of unique DNA or RNA sequences or specific genes, within the total DNA or RNA extracted from human or animal tissue, any indicate the presence of genetic diseases, of conditions such as sickle cell anemia, tissue compatibility, cancer and precancerous states, or bacterial or viral infections. The identification of unique DNA or RNA sequences or specific genes within the total DNA or RNA extracted from bacterial cultures or tissue containing bacteria may indicate the presence of antibiotic resistance, toxins, viruses, or plasmids, or provide identification between types of bacteria.

Thus, nucleic acid hybridization assays have great potential in the diagnosis and detection of disease. Further potential exists in agriculture and food processing where nucleic acid hybridization assays may be used to detect plant pathogenesis or toxin-producing bacteria.

One of the most widely used nucleic acid hybridization assay procedures is knows as the Southern blot filter hybridization method or simply, the Southern procedure (Southern, E., J. Mol. Biol. I. 98,503, 1975). The Southern procedure is used to identify target DNA or RNA sequences. This procedure is generally carried out by immobilizing sample RNA or DNA to nitrocellulose sheets. The immobilized sample RNA or DNA is contacted with radio-labeled probe strands of DNA having a base sequence complementary to the target sequence carrying a radioactive moiety which can be detected. Hybridization between the probe and the sample DNA is allowed to take place.

The hybridization process is generally very specific. The labeled probe will not combine with sample DNA or RNA if the two nucleotide entities do not share substantial complementary base pair organization standard. Hybridization can take from three to 48 hours depending on given conditions.

To the extent possible, unhybridized DNA probe is subsequently washed away. However, as a practical matter there is always nonspecific binding of the labeled probe to supports which appears as "background noise" on detection. Background noise reduces the sensitivity of an assay. The nitrocellulose sheet is placed on a sheet of X-ray film and allowed to expose. The X-ray film is developed with the exposed areas of the film identifying DNA fragments which have been hybridized to the DNA probe and therefore have the base pair sequence of interest.

The use of radioactive labeling agents in conjunction with Southern assay techniques have allowed the application of nucleic acid assays to clinical samples. Radioactive decay is detectable even in clinical samples containing extraneous proteinaceous and organic material. However, the presence of extraneous proteinaceous and organic material may contribute to nonspecific binding of the probe to the solid support. Moreover, the use of radioactive labeling techniques requires a long exposure time to visualize bands of X-ray film. A typical Southern procedure may require 1 to 7 days for exposure. The use of radioactive labeling agents further required special laboratory procedures and licenses.

The above problems associated with assays involving radioisotopic labels have led to the development of techniques employing nonisotopic labels. Examples of nonisotopic labels include enzymes, luminescent agents, and dyes. Luminescent labels emit light upon exitation by an external energy source and may be grouped into categories dependent upon the source of the exciting energy, including: radioluminescent labels deriving energy from high energy particles; chemiluminescent labels which obtain energy from chemical reactions; bioluminescent labels wherein the exciting energy is applied in a biological system; and photoluminescent or fluorescent labels which are excitable by units of electromagnetic radiation (photons) of infrared, visual or ultraviolet light. See, generally, Smith et al., Ann. Clin. Biochem., 18: 253 274 (1981).

Nonisotopic assay techniques employing labels excitable by nonradioactive energy sources avoid the health hazards and licensing problems encountered with radioisotopic label assay techniques. Moreover, nonisotopic assay techniques hold promise for rapid detection avoiding the long exposure time associated with the use of X-ray film.

However, nonisotopic assays have not conveyed the sensitivity or specificity to assay procedures necessary to be considered reliable. In luminescent assays, the presence of proteins and other molecules carried in biological samples may cause scattering of the exciting light or may absorb light in the spectrum of emission of the luminescent label, resulting in a quenching of the luminescent probe.

In enzymatic assays, the presence of proteins and other molecules carried in biological samples may interfere with the activity of the enzyme.

Similarly, in colorimetric assays, the change in color may not be detectable over proteins and other materials carried in biological samples.

Additionally, the time period for hybridization in Southern-like procedures may be unacceptable for some applications. The use of a two probe systems to effect target capture on conventional non-retrievable supports has been suggested in an article authored by Ann-Christine Syuänen, Matti Laaksonen and Hans Söderlund entitled "Fast Quantification of Nucleic Acid Hybrids by Affinity-Based Hybrid Collection;" Nucleic Acids Research, 14(12):5037 (1986), and U.S. Patent 4,751,177 to Stabinsky. The interest in multiple probe systems is in part an effort to improve the kinetics of hybridization.

Copending application U.S.S.N. 922,155, filed October 23, 1986 addresses issues of background, hybridization kinetics and kinetics with regard to binding target to supports. One embodiment of the invention of U.S.S.N. 922,155 comprises a method for isolating target. By way of exapmle, without limitation, one method comprises contacting a sample medium potentially containing target molecules with probes and a first support associated or capable of associating with at least one probe under binding conditions. The

probes are capable of selectively reversibly binding to the target molecule to form a complex including the probe target and the first retrievable support. Next, the support is separated from the sample medium and brought into contact with a second medium. Next, the support is subjected to releasing conditions to release the target from the support and the support is separated from the second medium. Next, a second support is contacted with the second medium under binding conditions. The second support is associated with or capable of associating with at least one probe capable of selectively binding to the target molecule. Under binding conditions, the target forms a complex with the probe associated to second support for further processing.

Embodiments described in U.S.S.N. 922,155 feature a first suppport which is retrievable in the sense that it is capable of substantially homogeneous dispersion within the sample medium and can be substantially physically separated, retrieved, or immobilized within the sample medium.

Separation of the first support from the first medium removes nonspecifically bound cellular debris attached to the first support. Further binding of the target molecule to a second support further concentrates the target for detection and permits further release-capture cycles for greater purification.

The binding or capture and subsequent release of a target for further processing is sometimes referred to as reversible target capture or RTC. One such capture and release is sometimes referred to as a cycle of RTC.

Powerful amplification techniques give rise to special problems. In some amplification chemistries, a single molecule can trigger a cascade resulting in signal. In an open laboratory environment, such molecules may exist in the environment carried by air or on equipment. Contamination on equipment can lead to sample

to sample, and assay to assay carryover. Molecules which are carried in the air due to microdroplets and turbulence can give rise to spurious results which are perhaps acceptable for research purposes but problematic for clinical applications. Open automated instrumentation, running countless assay procedures, may quickly become contaminated with such molecules and become inaccurate in their performance.

Summary of the Invention

Embodiments of the present invention feature a vessel for isolating target from a sample and for performing amplification, in a closed environment. Embodiments of the present invention are well suited for performing diagnostic procedures for detecting target molecules.

One embodiment of the present invention features a vessel for isolating target in a sample. The vessel comprises at least one reaction chamber, wash means and effluent means. The reaction chamber comprises a closed cell adapted to receive a support, a sample potentially containing target and at least one first probe, and thereafter being closed. The probe is capable of associating with the support and the target to form a support-probe-target complex and sample debris upon imposition of probe binding conditions within the reaction chamber. Wash means are capable of introducing solutions into the reaction chamber for washing the support to solubilize and suspend sample debris. Upon imposition of wash conditions, solutions are allowed to enter the reaction chamber to solubilize such sample debris. Effluent means are in communication with the reaction chamber and capable of receiving sample debris and wash solutions. The vessel receives the sample, binds the target, if present, to the support, allows wash solutions

to remove sample debris, and removes wash solutions and sample debris through effluent means, leaving target isolated on the support.

One embodiment of the present invention features wash means which comprise at least one wash chamber and at least one wash communication means. The wash chamber comprises a closed cell adapted to hold solutions in a closed environment for introduction into the reaction chamber. Wash communication means are interposed between the reaction chamber and the wash chamber to maintain such chambers and solutions separate until imposition of wash communication conditions. Upon imposition of wash communication conditions, solutions held in the wash chamber are allowed to enter the reaction chamber to solubilize and suspend sample debris. The wash chamber allows the vessel to be more self contained.

A still further embodiment of the present invention features effluent means comprising at least one effluent chamber and at least one effluent communication means. The effluent chamber comprises a compartment for receiving solutions from the reaction chamber. Effluent communication means are interposed between the reaction chamber and the effluent chamber and are capable of maintaining fluid in the effluent chamber and the reaction chamber substantially separate until the imposition of effluent communication conditions. By way of example, without limitation, one embodiment features effluent communication means comprising a passage, which passage is capable of cooperating with external clamps to restrict or open the passage.

A further embodiment of the present invention features a plurality of reaction chambers, wash means, effluent means, eluent means, and at least one reaction chamber communication means. The plurality of reaction chambers comprise a first reaction chamber and a second reaction chamber. The first reaction chamber comprises a

closed cell adapted to receive the support, sample potentially containing target, and probe. After receiving the support, sample and probe, the first reaction chamber is capable of being closed. The probe is capable of releasing from target to form a probe-target complex upon imposition of release conditions. Wash means are capable of introducing wash solutions into the first reaction chamber upon imposition of wash communication conditions to remove sample debris. Effluent means are capable of receiving sample debris and wash solutions from the first reaction chamber. Eluent means are capable of introducing solutions into the first reaction chamber upon imposition of eluent communication conditions at which eluent solutions held in the eluent chamber are allowed to enter the first reaction chamber to solubilize the probe-target complex. The second reaction chamber is comprised of a closed cell adapted to receive the eluent solution from the first reaction chamber. Reaction chamber communication means are interposed between the first and second reaction chambers and capable of maintaining the first and second reaction chambers separate until imposition of reaction chamber communication conditions at which time solutions held in the first reaction chamber are allowed to enter the second reaction chamber, leaving behind the support. The vessel allows a first reaction chamber to receive sample, and bind target to support, remove sample debris, and release target from the support for collection in the second reaction chamber.

With an emphasis on diagnostic application, one embodiment of the present invention features at least one reaction chamber which is capable of receiving a detection probe. The detection probe is capable of binding to target to form a detection probe-target complex the presence of which can be detected.

Similarly, for diagnostic applications, a further embodiment of the present invention features a read chamber and read chamber

communication means. The read chamber comprises a closed cell having read surfaces. The read chamber is capable of receiving solutions from a reaction chamber, which solutions, in the event target is present in the sample, are capable of producing a detectable response. The read surfaces are capable of transmitting such detectable response to the exterior of the vessel. The read chamber communication means is interposed between the read chamber and the reaction chamber and capable of maintaining the read chamber and the reaction chamber separate until imposition of read communication conditions. Upon imposition of read communication conditions, solutions held in the reaction chamber are allowed to enter the read chamber for detection purposes.

In one embodiment in the present invention, the read chamber is adapted to hold detection reagents. The detection reagents allow the detection probe to produce a detectable response in the event target is or was present in the sample. One such detection reagent includes the enzyme Q-Beta Replicase and such other compositions necessary for the replication of MDV-1-like molecules, a 221 base RNA ribonucleic acid which self replicates in the presence of the enzyme.

In one embodiment, the detection reagents are held in one or more reagent chambers. Each reagent chamber comprising a closed cell which is opened to the read chamber upon imposition of read communication conditions.

In order to facilitate loading the vessel, one embodiment of the present invention features a vessel having at least one sample well and at at least one sample communication means. The sample well is adapted for receiving sample and comprises an open container adapted to receive sample. The sample well is capable of cooperating with cap means to close the sample well. The sample well communication means is capable of transporting the sample to

the reaction chamber upon imposition of sample communication conditions. One embodiment features sample well communication means comprising a passage to the reaction chamber.

One embodiment of the present invention features a vessel having sample well housing, cap means and a closure plug. The sample well housing is an open container having receiving surfaces for a cap housing. The sample well housing defines open container adapted to receive sample, and cooperates with sample communication means to transport sample to the reaction chamber. Cap means comprise a cap housing capable of being slidably received in the sample well housing. The cap housing defines an open container having two ends, one of the ends having a breakable wall, and the other adapt end adapted to receive the closure plug. The closure plug is adapted to fit and seal the open end of the cap housing to contain the sample in a closed environment. The cap housing end having the breakable wall is adapted to be slidably received by the sample well housing and wherein the breakable wall breaks, to release sample into the reaction chamber.

A further embodiment of the present invention features at least one probe well and at least one probe communication means. The probe well comprises a housing defining a container adapted to receive probe. The probe housing is capable of cooperating with probe cap means to close the probe well. The probe communication means is capable of transporting probe to the reaction chamber upon imposition of probe communication conditions.

In one embodiment, the probe cap means comprises a probe cap housing capable of being slidably received by the probe well housing. The probe cap housing defines a closed container having one end having a breakable wall which end having the breakable wall is adapted to be received by probe well housing. Upon imposition of

probe communication conditions, the probe well housing breaks the breakable wall to release probe.

A further embodiment of the present invention features a single probe-sample cap which includes features of the probe cap and sample cap. The single probe-sample cap allows the remaining part of the vessel to be generic for a variety of tests. Thus, the probe-sample cap can be loaded with probes in a manufacturing process specific for a particular assay. A technician, nurse or physician would load the sample into the probe-sample cap housing and close the housing with the closure plug. The sample-probe cap would be fitted to the remaining vessel. The remaining vessel can be made generic and applicable for a variety of assays. Only the probes need to be modified for each test. Preferably, each cap and remaining vessel will have identification means such as coding bars for the type of assay and provision for the name of the patient or the nature of the test and date.

Embodiments of the present invention are easily manufactured from plastic film. Preferably, the chambers and features of the vessel are formed by welding two films of plastic. The preferred plastic film is manufactured by DuPont de NeMours and sold under the tradename Surlyn®. Surlyn® is an ionomer resin thermoplastic containing both covalent and ionic bonds. The ionic intercharge electrostatic forces are very powerful and thermally reversible at temperatures varying from 175°C to 290°C. Two films of Surlyn® plastic can be placed together during the manufacturing process with one of the films heat-formed to the shape required on a continuous speed production process and the second film laid on top. Permanent walls may be formed between the two films to form chambers, passages and seals. In addition, semipermanent walls can be formed to serve as burstable seals.

Walls and seals are made with heated filaments. The duration of heat, compressive pressure exerted on the two films, temperature of the filament and cooling time influence the nature of the wall.

Embodiments of the present invention feature various communication means comprised of burstable seals. For example, without limitation, embodiments of the present invention feature wash communication means, reaction cell communication means, eluent communication means, and read communication means. All such communication means can readily be made in Surlyn® plastic to comprise burstable seals between chambers.

In addition, permanent walls can be incorporated within the vessel during the performance of the assay to reclose chambers that have been spent.

Preferred embodiments of the present invention feature a plurality of reaction cells, wash chambers, eluent chambers and effluent chambers to facilitate multiple rounds of reversible target capture leading to a final reading of the assay result in a read chamber.

Embodiments of the present invention allow the performance of target isolation and amplification in a closed environment. Each assay vessel is closed to every other vessel thereby avoiding cross contamination between samples and assays. Embodiments of the present invention feature a vessel which does not require the interaction of any common equipment which would be used in contact with solutions with any other sample. Vessels in accordance with the present invention are suitable for use in instrumentation capable of performing manual functions. To facilitate manual operations, one embodiment of the present invention features a backing plate.

The use of films of Surlyn® and backing plates allows the vessel of the present invention to be readily manufactured in a continuous manner.

Other aspects and advantages of the invention will become apparent upon consideration of the following detailed descriptions with reference to Figures 1 through 9, which by way of example, illustrate preferred embodiments of the present invention and the principles thereof, and what is now considered to be the best mode to apply these principles.

Brief Description of the Drawings

Figure 1 is a exploded perspective view of a vessel and vacuum plate embodying features of the present invention.

Figure 2 is a vessel embodying features of the present invention adapted to receive a sample and perform four cycles of reversible target capture and produce a detectable response in the presence of target.

Figure 4 is a side perspective view of the vessel of Figure 2.

Figure 3 is a sectional side view of the vessel of Figure 2 illustrating the cooperation between a sample-probe cap and a sample and probe housings embodying features of the present invention.

Figure 5 is a top perspective view of the sample-probe cap embodying of Figure 2.

Figure 6 is a side cross-sectional view of the sample-probe cap illustrated in Figure 5.

Figure 7 is a bottom perspective view of the sample-probe cap embodying features of the present invention illustrated in Figure 5.

Figure 8 is a side perspective view of the sample-probe cap embodying features of the present invention as illustrated in Figure 5.

Figure 9 is a side corss-sectional view of the sample probe cap illustrated in Figure 5.

Detailed Descriptions of the Drawings

To facilitate discussion of the present invention, reference will be made to Figures 1 through 9, which illustrate preferred embodiments of the present invention adapted for use with nucleic acid probes for the detection of nucleic acid targets. Those skilled in the art will recognize the application of the invention for other ligand and antiligand systems as well. Turning first to Figure 1, Figure 1 shows, in exploded perspective view, a vessel 11, embodying features of the present invention. The vessel 11 is comprised of the following major elements: a backing plate, generally designated 13, a first sheet of plastic film 15 and a second sheet of plastic film 17. The two sheets of plastic film 15 and 17 are positioned one on top of the other. Alignment holes 19 in the first and second sheets 15 and 17 allow each plastic film to be stably positioned with respect to each other and with respect to the backing plate 13. Backing plate 13 is equipped with holes 21 or, in the alternative pins (not shown) capable of cooperating with holes 19. Adhesive can be used to secure the sheets 15 and 17 with respect to backing plate 13.

The two sheets of plastic film are preferably comprised of Surlyn®. The two sheets of plastic film, 15 and 17, are welded together to form features of the vessel 11. Heavier lines, depicted in Figure 1, are indicative of permanent seals or walls. Lighter lines are indicative of breakable seals or walls. The difference between breakable seals and permanent walls being one of degree rather than character. Permanent walls tend to retain integrity up to 50-60 psi. Burstable seals tend to rupture at pressures of between 10-30 psi. Such permanent seals or walls are made with a wider filament, which filament is capable of higher temperature,

held against the two sheets at higher compressive pressure, for a greater time and cooled more slowly than burstable seals.

The vessel illustrated in Figure 1 has a first reaction chamber generally designated by numeral 31. The first reaction chamber 31 is adapted to receive a support, a sample potentially containing target, and at least one first probe and thereafter being substantially closed. The probe is capable of associating with the support and the target to form a support-probe-target complex and sample debris upon imposition of probe binding conditions.

As illustrated, sample is introduced into first reaction chamber 31 through sample well 33. Sample well 33 is formed by the first and second sheets of plastic film 15 and 17. The first and second sheets of plastic film are blistered as generally illustrated by the shaded area 35, to provide for additional volume between the two sheets, to accommodate sample. When positioned on the backing plate 13, blister 35 is retained in part by a compartment 37 which allows the first sheet 15 to rest firmly in place.

Probe is added to the sample in the sample well 33 or may be present in the first reaction chamber 31. Addition of probe to sample well 33 allows the probe to form a probe-target complex, prior to binding to support, while the probe and sample are in the sample well 33. After sample has been introduced into the sample well 33, sample well 33 can be sealed to form a closed cell. Dotted line 37 generally represents a permanent seal welded between the first and second sheets of film 15 and 17 after the vessel has received probe and sample.

Following the formation of the permanent seal 37, solutions held in the sample well 33 are urged into the first reaction chamber 31 by sample communication conditions. Sample communication conditions comprise compression of sample well 33 forcing solutions through burstable seal 39. Burstable seal 39 maintains sample well

33 and first reaction chamber 31 separate until sample well 33 is compressed. Burstable seal 39, as do all burstable seals, includes a point area 39a which point area is directed against the flow of fluid. The point area 39a, due to its geometry, creates a weak point in the weld forming burstable seal 39, allowing such seals to break in a consistent manner.

After the fluids have been moved to the reaction chamber 31, a new seal can be welded into the first and second films of plastic 15 and 17 such that fluids cannot reenter the sample well 33. As illustrated, such further weld is generally depicted by dotted line 41 which weld defines the first reaction chamber 31 and forms a closed cell.

In assays which employ a two-probe system, wherein one detection probe is capable of producing a detectable response, and a second capture probe is capable of capturing a detectable probe-target complex to a solid support, it is advantageous to allow the sample and the capture probe to hybridize prior to capture on supports. Capture of the capture probe on the support prior to the capture probe hybridization to target, impairs the kinetics of the binding of probes to target, causing a lower duration in hybridization. The present vessel 11 facilitates hybridization of the probes to the target by maintaining the support separate from the sample during an initial hybridization. Such hybridization may take place in sample well 33 or the first reaction chamber.

Following hybridization, magnetic supports or magnetic beads are preferably received within the first reaction chamber 31. As illustrated, magnetic supports in the form of beads are contained within a support chamber 43. Support chamber 43 is divided into two parts, an upper support chamber, 43a, and a lower support chamber 43b. In order to facilitate the filling of support chamber 43a and 43b, the vessel 11 is equipped with filling chambers 45a and 45b,

which allow the vessel 11 to cooperate with filling nozzles, funnels and the like (not shown). After the supports have been loaded into the support chamber 43a and b, the filling chambers 45a and 45b are no longer needed. A permanent seal is welded between the upper and lower plastic sheets at the dotted line 47, sealing the support within the support chambers 43a and 43b which become closed cells. Support chambers 43a and 43b are blistered in order to increase their capacity to hold magnetic supports. Backing plate 13 has indentations 61a and 61b to receive the blisters of support chambers 43a and 43b.

One embodiment of the invention features magnetic supports characterized in their ability to be substantially homogeneously dispersed in a sample medium. Preferably, the magnetic beads carry primary amine or carboxyl functional groups which facilitate covalent binding or association of a probe entity to the magnetic support particles. Preferably, the magnetic support beads are single domain magnets and are superparamagnetic exhibiting no residual magnetism. As described herein, the magnetic support is capable of substantially homogeneous dispersion within the sample medium and includes at least one antiligand moiety capable of binding to a ligand under binding conditions to form a target-probe support complex.

After the target and the probes have been allowed to hybridize under binding conditions within reaction chamber 31, magnetic supports held in support chamber 43a and support chamber 43b are urged into the reaction chamber 31 by imposition of support communication conditions. Support communication conditions include compressing, manually or by equipment, support chambers 43a and 43b to burst the burstable seal 49. Burstable seal 49 is interposed between the magnetic support chamber 43a and 43b and reaction chamber 31 maintaining such chambers separate and apart from each

other until such compressive force is exerted on support chambers 43a and 43b. After the magnetic support has entered the reaction chamber 31, support chambers 43a and 43b are sealed from the reaction chamber 31, to avoid fluids backing up into such chambers, by the formation of a permanent seal along dotted line 89a.

The magnetic supports which are now present in reaction chamber 31 are mixed to provide intimate contact and dispersion within the solutions retained in reaction chamber 31. The vessel 11 comprised of flexible plastic films 15 and 17, allows for mixing of solutions by simply rolling across the body of the bag-like structure formed.

The probe-target complex is allowed to bind to the magnetic support, and the magnetic support is immobilized. Backing plate 13 is equipped with two hollowed out areas 63a and 63b, towards the back side of the plate to allow the magnets (not shown) used for immobilizing the magnetic supports to be positioned in close proximity to such supports. The use and need for such magnetic support indentations 63a and 63b is dependent upon the thickness of the backing plate 13 and the strength of magnets used for immobilization. During immobilization, sample debris is separated from the target-probe support complex by compressing reaction chamber 31 to urge solutions through effluent passage 69 and into effluent chamber 67. Effluent passage 69 is interposed between the effluent chamber 67 and the first reaction chamber 31. Preferably, effluent passage 69 is kept in a substantially closed position until the imposition of effluent communication conditions. Effluent passage 69 can be maintained in a closed position by compressive clamping pressure on the passage 69 by manual or mechanical means.

Effluent chamber 67 is formed by welding the first and second plastic films 15 and 17 to form a closed cell. Effluent chamber 67 is blistered to accommodate retention of fluids. In order to

accommodate the blistered effluent chamber 67, backing plate 13 has an indentation or cutout 71. Following removal of solutions containing sample debris from the first reaction chamber, the magnetic supports and the walls of reaction chamber 31 may contain further nonspecifically bound sample debris. In order to solubilize or suspend such further sample debris, the magnetic supports are further washed.

A series of ~~three~~ wash chambers 73a, 75a and 77a are welded into the first and second plastic films. Each wash chamber 73a, 75a and 77a is blistered to accommodate wash solution volumes. Each wash chamber 73a, 75a and 77a has a filling chamber 73b, 75b and 77b respectively. Each filling chamber is also blistered to accommodate nozzles, tubes, funnels and other filling apparatus (not shown). After the filling operation is complete and fluids are retained within the ~~first, second and third~~ wash chambers 73a, 75a and 77a, each wash chamber is sealed by the formation of a permanent seal along dotted line 47 to form closed cells.

In order to accommodate the blistered wash chambers 73a, 75a and 77a, backing plate 13 has indentations generally designated by numerals 81, 82 and 83 adapted to receive each blister. Each wash chamber 73a 75a and 77a, is maintained separate from the reaction chamber 31 by a burstable seal 87a, b and c. Upon imposition of wash communication conditions on the first wash chamber 73a, wash breakable seal 87a opens and allows the solutions retained within the wash chamber 73a to enter the reaction chamber 31. Upon suitable mixing, the magnetic supports are immobilized within the first reaction chamber 31 and the wash solutions removed through effluent passage 69 into effluent chamber 67.

In order to avoid back flow of solutions into any of the open wash chambers 73a, 75a and 77a, each chamber can be sealed after

solutions have been removed therefrom by forming a permanent seal along by dotted line 89b, c and d.

Following the wash from the solutions in the first wash chamber 75a, the supports retained in reaction chamber 31 are washed sequentially with the solutions retained in the second wash chamber 75a and, in a similar manner, the wash solutions retained in the third wash chamber 77a. During each wash, magnetic supports are retained in the reaction chamber 31 and wash solutions are removed from the reaction chamber 31 by effluent passage 69 into effluent chamber 67.

Preferably, the antiligand system of the support and probe are reversible to allow a probe-target complex to be removed from the magnetic support. Embodiments of the present invention feature complementary homopolymer nucleic acids, ligands and antiligands. One homopolymer nucleic acid is associated with a magnetic particle and a complementary homopolymer nucleic acid is associated with the probe. Such homopolymers can be released upon suitable release conditions such as alterations in temperature, pH, salt strength and the like.

Eluent chamber 90a contains solutions which facilitate dissolution of the target-probe complex from the support. Vessel 11 has a filling chamber 90b to facilitate nozzles, funnels, and the like useful for injecting solutions within the chamber 90a. Eluent chamber 90a and eluent filling chamber 90b are formed by welding the first and second plastic films.

After solutions are placed in the eluent chamber 90a, eluent chamber 90a is sealed by the formation of a permanent seal along dotted line 47 to form a closed cell. Eluent filling chamber 90b, as well as wash filling chambers 73b, 75b and 77b, and support filling chamber 45a and 45b are no longer required after filling, for the further functioning of the vessel 11, and can be removed.

Eluent chamber 90a and filling chamber 90b are blistered to accommodate the solutions and filling apparatus (not shown). Backing plate 13 has a cooperating indentation 85 adapted to receive the blister.

In order to accommodate the blistered area of eluent chamber 90a, backing plate 13 is equipped with an indentation 91.

Eluent chamber 90a is maintained separate from reaction chamber 31 by a burstable seal 93. Upon imposition of eluent communication conditions, burstable seal 93 opens to release the eluent solutions retained within the chamber 90a into reaction chamber 31. Eluent release conditions comprise compressing eluent chamber 90a to urge solutions through the burstable seal 93. In order to prevent such solutions from back-flowing, a permanent seal can be placed by welding the first and second sheets films of plastic 15 and 17 along dotted line 89e. Upon imposition of release conditions, the target-probe complex is released from the support, into the eluent solutions.

The magnetic supports used within the first reaction chamber 31, and the reaction chamber 31 itself, may hold unacceptable levels of nonspecifically bound probe and sample debris. In solution, the target-probe complex is removed from the first reaction chamber 31 through burstable seal 95 into a second reaction chamber 97. The second reaction chamber 97 formed between welds of the first and second sheets of film 15 and 17. A new permanent seal can be formed about burstable seal 95 to form a closed cell.

Second reaction chamber 97 is blistered to accommodate fluid volumes. Backing plate has an indentation 98 to receive and hold blistered second reaction chamber 97.

The target-probe complex retained within the second reaction chamber 97 can be further processed for detection steps, or amplification, or any other process requiring a substantially

isolated target. In the event that such target is desired outside the vessel, a final burstable seal 99 is opened for removal of the contents of the second reaction chamber 97.

Further processing of the target-probe complex may include additional cycles of reversible target capture. A further embodiment of the present invention features four reversible target cycles.

Figures 2 through 4 illustrate a vessel generally designated by the numeral 111 embodying features of the present invention. The vessel 111 is comprised of the following major elements: a backing plate 113, a first sheet of plastic film 115, a second sheet of plastic film 117, and a cap 123. The first sheet of plastic film 113 is positioned underneath a second plastic sheet 117 in Figure 2 and, therefore, is obscured from view.

The relationship of cap 123 to the backing plate 113, can best be seen in Figure 3. The cap 123 is illustrated in greater detail in Figures 5 through 8.

Turning first to cap 123, cap 123 includes a housing 125 which is molded with a first probe containment area 127a, a second probe containment area 127b, and a sample containment area 128. The first probe containment area 127a, second probe containment area 127b and the sample containment area are cylindrical in shape and have two circular ends. As best seen in Figure 6 and Figure 7, one end of each probe containment area 127a and 127b is enclosed by a breakable wall of plastic or foil represented by the numeral 130. The other end of first probe containment area 127a and second probe containment area 127b is comprised of a foil seal 129. Foil seal 129 is affixed to the housing 125 after the probe solutions are placed within the first probe containment area 127a and the second probe containment area 127b.

Sample containment area 128 has two ends in which one end is covered by foil seal 129, as best seen in Figure 7, and the remaining end is open to receive sample, as best seen in Figures 6 and 9. After the sample has been placed in sample containment area 128, sample plug 131 is placed over the sample containment area and received by the cap housing 125 to sealably contain the sample within the sample containment area 128. Sample plug 131 has a breakable wall of plastic or foil 613.

Figure 8 includes a side perspective view of cap housing 125 in which a bar code 132 has been imprinted to facilitate reading of the cap type by an instrumentation reader device.

Turning now to Figure 3, backing plate 113 includes a well housing 133. Well housing 133 is capable of receiving cap housing 125. Well housing 133 includes a first probe well 134a and a second probe well 134b. Each probe well 134a and 134b has a central opening defining a passage for the movement of fluid. Probe well 134a and b project upwardly and are pointed to break seals 130 as the cap housing is pushed down into the well housing 133. The seals 130 pulled over and against the probe wells 134a and b to seal against the projection.

Similarly, well housing 133 includes a sample well 135 having a central opening defining a passage for the movement of fluid. Sample well 135 projects upwardly from the well housing 133 and is pointed in configuration to facilitate breaking seal 613 as the cap housing is pushed downward on the well housing 133.

As cap housing 125 is received within the well housing 133, fluids contained within the first probe containment area 127a and a second probe containment area 127b, and sample contained within the sample containment area 128 are urged through the passages of probe well 134a and b and the passage within sample well 135.

Cylindrical projections 228 extending around sample well 135 and probe wells 134a and 134b cooperate with the cylindrical forms defining containment areas 128 and 127a and to maintain and seal fluids within the containment areas and urge fluids through passages.

As illustrated, sample flows through passages of sample well 135 into a blister generally designated as 137. Probes solutions contained within the first probe area 127a flow through passages of probe well 134a into a first probe blister 138. Probe solutions contained with the second probe containment area 127b flow through probe well 134b into a second probe blister 139. After the probe and sample are placed in respective blisters, the sample well 135 and probe 134a and b can be sealed from the blisters by forming a permanent seal (not shown).

Vessel 111 has a first reaction chamber 141 formed by welding permanent walls 119 within first sheet of plastic 115 and second sheet of plastic 117. Sample and first probe solutions contained within the first probe blister 138 and sample blister 137 are urged into the first reaction chamber 141 by compressing the blister and forcing the solutions contained therein through burstable seals. Sample blister 137 is maintained separate and apart from first reaction chamber 141 by a burstable seal 142 and first probe blister is maintained separate from reaction chamber 141 by burstable seal 143. After the first probe solution and sample have entered the first reaction chamber, the first probe blister 137 and sample blister 138 can be sealed from the first reaction chamber 141 by the formation of a permanent seal (not shown).

Under hybridization conditions, the probes contained within the first probe solution are capable of binding to target. The vessel 111 contains magnetic supports to capture the probe-target complex formed. Such magnetic beads are maintained separate from the first reaction chamber 141 in a support chamber 145. Support

chamber 145 includes two blistered areas to contain the bulk of the support. The support chamber 145 is maintained separate from first reaction chamber 141 by a burstable seal 147. Supports contained within the support chamber 145 are urged into the first reaction chamber 141 by compressing the chambers to urge the supports through the burstable seal 147. Target, if present, probe and support forms a target-probe support complex. The magnetic supports are immobilized about the back sheet 115 and backing plate 113 by means of magnets positioned about the backing plate at positions 148a and 148b. The solutions containing sample debris are removed from the first reaction chamber 141 while the magnetic supports are immobilized. The solutions containing sample debris are urged by compressing the first reaction chamber 141 to urge solutions through passage 149 connecting the first reaction chamber 141 to effluent chamber 150.

In order to prevent back flow of solutions from the effluent chamber 150 to the first reaction chamber 141, the effluent passage 149 can be maintained closed by compressing the passage or clamping the passage.

Although much sample debris is removed by the first removal of solutions from the reaction chamber 141, sample debris may still be present in the first reaction chamber 141, nonspecifically bound to the supports and the internal structures of the chamber. In order to solubilize and suspend such further sample debris, wash solutions contained in a first wash chamber 151 are urged upon imposition of wash communication means, into the first reaction chamber 141. Wash chamber 151 is maintained separate from the first reaction chamber by means of a burstable seal 151a. Imposition of wash communication means comprises compression of the wash chamber blister 151 to urge such solutions through burstable seal 151a.

Magnetic supports containing the target-probe complex are released from the backing plate and bottom plastic sheet 115, in order to facilitate intimate contact with the wash solutions. The wash solutions are mixed with the magnetic supports by rolling solutions from end to end in the reaction chamber with a light pressure on the top plastic sheet 117. After the wash solutions have been thoroughly mixed throughout the reaction chamber 141 and the supports, the magnetic supports are again immobilized in areas 148a and 148b. Wash solutions are removed from the first reaction chamber 141 through passage 149 and into effluent chamber 150.

The magnetic supports are released and solutions from a second wash chamber 152 are allowed to enter first reaction chamber 141. Wash solutions maintained in second wash chamber 153 are urged into the first reaction chamber 141 through a burstable seal 152a by compressing the blister. Following suitable mixing, the magnetic supports maintained in first reaction chamber 141 are again immobilized and solutions removed through passage 149 to effluent chamber 150.

Similarly, the magnetic supports are released and solutions from a third wash chamber 153 are allowed to enter first reaction chamber 141. Wash solutions maintained in third wash chamber 153 are urged into the first reaction chamber 141 through a burstable seal 153a by compressing the blister. Following suitable mixing, the magnetic supports maintained in first reaction chamber 141 are again immobilized and solutions removed through passage 149 to effluent chamber 150.

In order to separate the target and probe complex from the support, vessel 111 carries eluent solutions within an eluent chamber 154. Eluent chamber 154 is maintained separate from first reaction chamber 141 by a breakable seal 154a. Compresssion of the eluent chamber 154 urges eluent solutions through the burstable seal

154a and into the first reaction chamber 141. Eluent solutions are mixed with the magnetic support by rolling the solutions throughout the first reaction chamber 141. Under appropriate release conditions, the target-probe complex is released from the support.

Once again, the magnetic supports are immobilized in areas generally designated by the numeral 148a and 148b. Solutions carrying the target-probe complex are urged through a breakable seal 155 separating the first reaction chamber 141 from a second reaction chamber 241.

Second reaction chamber 241 can be maintained separate from the first reaction chamber 141 by formation of a permanent wall or seal generally in the area of breakable seal 155. Second reaction chamber 241 has many features which operate in a manner identical to that described with respect to the first reaction chamber 141. Second reaction chamber 241 is in communication with a eluent chamber 250 by means of a effluent passage 249. Magnetic supports for introduction into the second reaction chamber 241 are held in a support chamber 245. Breakable seals 247 maintain the support chambers 245 separate and apart from second reaction chamber 241. Backing plate 113 includes two areas for cooperation with magnets for immobilizing magnetic supports generally designated by areas 248a and 248b. First, second and third wash chambers, respectively, are generally designated by numerals 251, 252 and 253 are maintained separate and apart from the second reaction chamber 241 by means of burstable seals 251a, 252a and 253a.

Solutions held in probe chamber 139 can be urged into the second reaction chamber 241 through breakable seal 140. In two-probe systems, there may be less background where the detection probe is introduced in the second reaction chamber. In the present discussion, reference to a probe-target complex is meant to include

multiple probes which can be in such soltuions of probe chamber 139 and probe solutions of probe chamber 138.

Following recapture of the target-probe complex on supports and subsequent washes with wash solutions maintained in first, second and third wash chambers 251, 252 and 253, removal of solutions through passage 249 into effluent chamber 250, the target-probe complex can be released from the second support by eluent solutions maintained in an eluent chamber 254 in a manner as that described with respect to the first reaction chamber 141. Eluent chamber 254 is maintained separate from the second reaction chamber 241 by means of burstable seal 254a.

As with the first reaction chamber, following removal of solutions from wash chambers and eluent chambers, such chambers can be permanently sealed to avoid back flow of solutions into such chambers.

Eluent solutions carrying the target-probe complex released from the support are urged through a second chamber breakable seal 255 into a third reaction chamber 341. After solutions have entered third reaction chamber 341, third reaction chamber 341 can be closed to second reaction chamber 241 by formation of a permanent wall or seal at or about the breakable seal 255.

As with the second reaction chamber, the third reaction chamber is in communication with an effluent chamber 350 through an effluent passage 349. Magnetic supports are maintained in a third support chamber 345 and enter the third reaction chamber through a burstable seal 347. A first, second and third wash chamber are maintained separate from the third reaction chamber by burstable seal 151a, 152a and 153a. An eluent chamber 354, is maintained separate and apart from the third reaction chamber 341 by burstable seal 354a. The function and operation of such components and elements is identical to the second reaction chamber. Following

capture of the target-probe complex on a support and subsequent washes, the target-probe complex is released from the support. Eluent solutions containing the target-probe complex are urged through burstable seal 355 by compressing the third reaction chamber 341.

Eluent solutions containing the target-probe complex pass through burstable seal 355 into a fourth reaction chamber 441. Reaction chamber 441 is similar in structure and operation to second reaction chamber 241 and third reaction chamber 341. Reaction chamber 441 is in communication with a fourth effluent passage 449 communicating with effluent chamber 450. Magnetic supports are maintained in support chamber 445 separate and apart from the fourth reaction chamber 441 by burstable seal 447. Such supports can be immobilized within areas generally designated 448a and 448b with cooperating magnets (not shown). Wash solutions are maintained in blistered chambers 441, 452 and 453 separate and apart from the fourth reaction chamber 441 by burstable seals 451a, 452a and 453a. Eluent solutions for solubilizing the target-probe complex after washes are maintained in blistered eluent chamber 454 separate and apart from the fourth reaction chamber by means of a burstable seal 454a. In the event additional washes or eluent solutions are desired, they may be carried in extra chambers 460, 461 and 462.

Following capture on supports, washes and elutions from such supports, the elution solution held in fourth reaction chamber 441 is forced through breakable seal 455 into a fifth reaction chamber 541. In order to maintain fifth reaction chamber 541 separate and apart from fourth reaction chamber 441, a permanent seal can be welded into the first and second sheets 115 and 117 along the area of burstable seal 455.

Preferably, one of the probes has a label capable of detection. Reagents to facilitate production of a detectable

response are carried in detection chambers 571 and 572. In the event that the probe includes a replicatable entity, such as MDV-1-like sequences, the detection chambers 571 and 572 contain the enzyme Q-Beta replicase and necessary cofactors and agents.

Fifth reaction chamber includes a read surface 575. Read surface 575 is maintained separate from fifth reaction chamber 541 by a burstable seal 576 in order to allow complete mixing of the reagents prior to contacting the read surface 573 with the solutions. Complete mixing prior to reading signal will produce more consistent readings.

In the event that the probes include MDV-1 like sequences that are replicated by the enzyme Q-Beta replicase during a detection phase, one of the reagents of detection may include propidium iodide. Read surface 575 allows fluorescent detection of propidium iodide outside the fifth reaction chamber 541.

Embodiments of the present invention as illustrated in Figures 3 through 4 are capable of performing four reversible target capture cycles in a closed environment with amplification of a detectable moiety. The signal from the detectable moiety can be detected external of the vessel 111. Each vessel is contained. Solutions and apparatus for each assay are unique and are not comingled with any other assay.

While preferred embodiments have been illustrated and described, it is understood that the present invention is capable of variation in modification and, therefore, should not be limited to the precise details set forth but should include such changes and alterations that fall within the purview of the following claims.

1. A vessel for isolating target in a sample comprising: at least one reaction chamber, wash means, and effluent means; said reaction chamber comprising a closed cell adapted to receive a support, a sample potentially containing target, and at least one first probe and thereafter being substantially closed, said probe capable of associating with said support and said target to form a support-probe-target complex and sample debris upon imposition of probe binding conditions within said reaction chamber; said wash means capable of introducing solutions into the reaction chamber for washing said support to solubilize and suspend sample debris; upon imposition of wash conditions, at which time solutions are allowed to enter said reaction chamber; and, said effluent means in communication with said reaction chamber to receive sample debris and wash solutions; said vessel for receiving sample, binding target to said support, removing sample debris and washing said support to isolate target.

2. The vessel of claim 2 wherein said wash means comprises at least one wash chamber and at least one wash communication means; said wash chamber comprising a closed cell adapted to hold solutions in a closed environment for introduction into the reaction chamber; said wash communication means, interposed between, said reaction chamber and said wash chamber and capable of maintaining said wash chamber separate from said reaction chamber until imposition of wash communication conditions, at which time solutions held in said wash chamber are allowed to enter said reaction chamber to facilitate maintaining a closed environment in the reaction cell.

3. The vessel of claim 1 wherein said effluent means comprises at least one effluent chamber and at least one effluent communication means, said effluent chamber comprising a compartment for receiving solutions from said reaction chamber; said effluent communication means interposed between said reaction chamber and said effluent chamber and capable of maintaining fluids in said effluent chamber and fluids in said reaction chamber separate until imposition of effluent communication conditions.

4. The vessel of claim 1 further comprising a plurality of reaction chambers, eluent means, and at least one reaction chamber communication means; said plurality of reaction chambers comprising at least one first reaction chamber and at least one second reaction chamber, said first reaction chamber comprising a closed cell adapted to receive said support, said sample potentially containing target and said probe, and thereafter being substantially closed, said probe capable of releasing from target to form a probe-target complex on imposition of release conditions; said wash means introduce wash solutions for washing said support in said first reaction chamber upon imposition of wash conditions; said eluent means capable of introducing eluent solutions into said first reaction chamber upon imposition of eluent conditions at which eluent solutions held in said eluent chamber are allowed to enter said first reaction chamber to solubilize said probe-target complex upon imposition of release conditions; said second reaction chamber comprising a closed cell adapted to receive said eluent solution from said first reaction chamber; and, said reaction chamber communication means interposed between said first and second reaction chamber and capable of maintaining said first and second reaction chambers separate, until imposition of reaction chamber communication conditions at which time solutions held in said first

reaction chamber are allowed to enter said second reaction chamber leaving behind said support, said vessel for receiving sample, binding target to said support, removing sample debris and washing said support, releasing said target from the support and collecting target in said second reaction chamber.

5. The vessel of claim 4 wherein at least one of reaction chamber is capable of receiving a detection probe which detection probe is capable of binding to target to form a detection probe-target complex, the presence of which can be detected.

6. The vessel of claim 5 further comprising a read chamber and read chamber communication means; said read chamber comprising a closed cell having read surfaces, said read chamber capable of receiving solution from one of said reaction chamber, which solutions, in the event target was present in said sample, are capable of producing a detectable response, and said read surfaces capable of transmitting said detectable response to the exterior of said read chamber, said read chamber communication means interposed between said read chamber and said reaction chamber and capable of maintaining said read chamber and said reaction chamber separate until imposition of read communication conditions, at which solutions held in said reaction chamber are allowed to enter said read chamber.

7. The vessel of claim 6 wherein said read chamber is adapted to hold detection reagents, which detection reagents produce a detectable response in the event target is present in said sample.

8. The vessel of claim 7 wherein said detection reagent comprises the enzyme Q-Beta replicase.

9. The vessel of claim 7 wherein said reagents are held in one or more reagent chambers, said reagent chamber comprising a closed cell which is opened to said read chamber upon imposition of read communication conditions.

10. The vessel of claim 1 further comprising at least one sample well and at least one sample communication means, said sample well for receiving sample and comprising an open container adapted to receive and hold sample and capable of cooperating with cap means to close said sample well, said sample well communication means capable of transporting sample to said reaction chamber upon imposition of sample communication conditions.

11. The vessel of claim 10 further comprising cap means, and closure plug; said cap means having a cap housing capable of being slidably received in said well housing, said cap housing defining an open container having two ends, one of said ends adapted to receive a closure plug and said other end having a wall, said closure plug having a wall to contain said sample in a closed environment, at least one of said walls being breakable and adapted to be received by said well housing to break said breakable wall to release sample into said reaction chamber.

12. The vessel of claim 1 further comprising at least one probe well and at least one probe communication means, said probe well for receiving probe and comprising probe well housing defining a container adapted to receive and hold probe, said probe housing capable of cooperating with probe cap means to close said probe well, said probe communication means capable of transporting probe to said reaction chamber upon imposition of probe communication conditions.

13. The vessel of claim 12 further comprising probe cap means; said probe cap means having a cap housing capable of being slidably received in said probe well housing, said cap housing defining closed container having one breakable wall, said end having said breakable wall adapted to be received in said probe well housing breaking said breakable wall to release probe.

14. The vessel of claim 1 further comprising at least one probe well, one probe communication, one sample well and one sample communication means; said probe well for receiving probe and comprising a probe well housing defining a container adapted to receiva and hold probe, said probe housing capable of cooperating with probe cap means close said probe well, said probe communication means capable of transporting probe to said reaction chamber upon imposition of probe communication conditions; said sample well for receiving sample and comprising a sample well housing defining an open container adapted to receive and hold sample incapable of cooperating with cap means to close the sample well, said sample well communication means capable of transporting sample to said reaction chamber upon imposition of sample communication conditions.

15. The vessel of claim 14 further comprising cap means, comprising a cap housing, said cap housing having a probe housing and a sample housing, said cap housing defining a closed container having one breakable wall, and having said breakable wall adapted to be received by said probe well housing to break said breakable wall and release probe, said sample housing defining a open container having two ends, one of said ends having a breakable wall, and said other end closed to contain said sample in a closed environment, said sample housing adapted to be slidably received by said well housing breaking said breakable wall to release sample into said reaction chamber.

16. The vessel of claim 1 comprising two films of plastic heat formed to form said reaction chamber.

17. The vessel of claim 2 wherein said wash communication means includes a burstable seal between said wash chamber and said reaction chamber.

18. The vessel of claim 4 wherein said eluent means includes an eluent chamber and at least one eluent chamber communication means; said eluent chamber comprising a closed cell adapted to hold solutions in a closed environment for introduction into the reaction chamber; said eluent communication means interposed between said reaction chamber and said eluent chamber maintaining said eluent chamber seperate from said reaction chamber until imposition of eluent communication conditions, at which solutions held in said eluent chamber are allowed to enter the reaction chamber to facilitate release of target-probe complexes.

19. The vessel of claim 16 wherein said two films of plastic are affixed to a backing plate.

20. The vessel of claim 16 wherein said films of plastic are comprised of Surlyn.

21. A vessel for isolating target in a sample comprising two films of plastic welded to form at least one reaction chamber, at least one wash chamber, effluent means, and at least one wash burstable seal; said reaction chamber comprising a closed cell adapted to receive a support, a sample potentially containing target, and at least one first probe and thereafter being closed, said probe capable of associating with said support and said target to form a support-probe-target complex and sample debris upon imposition upon probe binding condition within said reaction chamber; said wash chamber comprising a closed cell adapted to hold solutions in a closed environment for introduction into the reaction chamber; said wash burstable seal interposed between said reaction chamber and said wash chamber and capable of maintaining said wash chamber separate from said reaction chamber until imposition of compressive forces which burst such seal releasing wash solutions contained within the wash chamber into the reaction chamber; and, said effluent means in communication with the reaction chamber to receive sample debris and wash solutions from said reaction chamber; said vessel for receiving sample, binding target to said support, removing sample debris and washing said support to isolate target.

22. The vessel of claim 21 wherein said effluent means comprise at least one effluent chamber formed in the two films of plastic by heat sealing to form a closed cell capable of receiviing solution from said reaction chamber.

23. The vessel of claim 21 further comprising a plurality of reaction chambers, at least one eluent chamber and eluent burstable seal; said reaction chambers, eluent chamber and eluent burstable seal formed of said two films of said plastic; said plurality of reaction chambers comprising at least one first reaction chamber and at least one second reaction chamber, said first reaction chamber comprising a closed cell adapted to receive said support, said sample potentially containing target and said probe, and thereafter being closed, said probe capable of releasing from target to form a probe-target complex on imposition of release conditions; said wash chamber adapted to hold solutions for washing said support in said first reaction chamber; said eluent chamber formed with said two films of plastic, welded to form a closed cell, and capable of containing eluent solutions; said eluent burstable seal interposed between said first reaction chamber said eluent chamber and capable of breaking upon imposition of eluent communication conditions to introduce eluent solutions into said first reaction chamber to solubilize a probe-target complex; said second reaction chamber comprising a closed cell formed of two films of said plastic and adapted to receive said eluent solution from said first reaction chamber; said reaction chamber burstable seal interposed between said first and second reaction chamber and capable of maintaining said first and second reaction chamber separate until such imposition of reaction chamber communication conditions in which said burstable seal breaks and releases solutions in said first reaction chamber into said second reaction chamber leaving behind said support; said vessel for receiving sample, binding target to said support, removing sample debris and washing said support, releasing said target from the support and collecting target in said second reaction chamber.

24. The vessel of claim 23 wherein at least one of said reaction chambers is capable of receiving at least one detection probe which detection probe is capable of binding to target to form a detection probe-target complex, the presence of which can be detected.

25. The vessel of claim 24 further comprising a read chamber and read chamber communication means; said read chamber comprising a closed cell having read surfaces, said read chamber capable of receiving solution from one of said reaction chambers, said solutions, in the event target was present in said sample, are capable of producing a detectable response, and said read surfaces are capable of transmitting said detectable response to the exterior of said read chamber, said read chamber burstable seals interposed between said read chamber and said reaction chamber and capable of maintaining said read chamber and said reaction chamber separate until imposition of read communication conditions, at which solutions held in said reaction chamber are allowed to enter said read chamber.

26. The vessel of claim 25 wherein said read chamber is adapted to hold detection reagents which detection reagents produce a detectable response in the event target was present in said sample.

27. The vessel of claim 26 wherein said detection reagents comprise the enzyme Q-Beta replicase.

28. The vessel of claim 25 wherein said reagents are held in at least one reagent chambers, said reagent chamber comprising a closed cell which is opened to said read chamber upon imposition of read communication conditions.

29. The vessel of claim 26 wherein said read chamber is formed of rigid transparent plastic.

30. A vessel for isolating target in a sample comprising two films of plastic welded to form a plurality of reaction chambers, at least one reaction chamber burstable seal, at least one wash chamber, at least one wash burstable seal, at least one eluent chamber, at least one eluent burstable seal, and effluent means; said plurality of reaction chambers comprising at least one first reaction chamber and at least one second reaction chamber, said first reaction chamber comprising a closed cell adapted to receive a support, a sample potentially containing target and a probe, and thereafter being closed, said probe capable of associating with said support and said target to form a support-probe-target complex and sample debris upon imposition upon probe binding condition within said first reaction chamber and said probe capable of releasing from target to form a probe-target complex on imposition of release conditions; said wash chamber comprising a closed cell adapted to hold solutions in a closed environment for introduction into the first reaction chamber; said wash burstable seal interposed between said first reaction chamber and said wash chamber and capable of maintaining said wash chamber separate from said reaction chamber until imposition of compressive forces which burst such seal releasing wash solutions contained within the wash chamber into the first reaction chamber; said eluent chamber formed with said two films of plastic, welded to form a closed cell, and capable of containing eluent solutions; said eluent burstable seal interposed between said first reaction chamber and said eluent chamber and capable of breaking upon imposition of eluent communication conditions to introduce eluent solutions into said first reaction chamber to solubilize a probe-target complex; said second reaction

chamber comprising a closed cell, adapted to receive said eluent solution from said first reaction chamber; said reaction chamber burstable seal interposed between said first and second reaction chamber and capable of maintaining said first and second reaction chamber separate until such imposition of reaction chamber communication conditions in which said burstable seal breaks and releases solutions in said first reaction chamber into said second reaction chamber leaving behind said support; and, said effluent means in communication with said first reaction chamber to receive sample debris and wash solutions from said first reaction chamber; said vessel for receiving sample, binding target to said support, removing sample debris and washing said support, releasing said target from the support and collecting target in said second reaction chamber.

ABSTRACT OF THE INVENTION

Present invention features a vessel for isolating target in a sample. The vessel comprises at least one reaction chamber, wash means and effluent means. The reaction chamber comprises a closed cell adapted to receive a support, a sample potentially containing target and at least one first probe, and thereafter being closed. The probe is capable of associating with the support and the target to form a support-probe-target complex and sample debris upon imposition of probe binding conditions within the reaction chamber. Wash means are capable of introducing solutions into the reaction chamber for washing the support to solubilize and suspend sample debris. Upon imposition of wash conditions, solutions are allowed to enter the reaction chamber to solubilize such sample debris. Effluent means are in communication with the reaction chamber and capable of receiving sample debris and wash solutions. The vessel receives the sample, binds the target, if present, to the support, allows wash solutions to remove sample debris, and removes wash solutions and sample debris through effluent means, leaving target isolated on the support.

Avoiding false positives with PCR

S. Kwok and R. Higuchi

The exquisite sensitivity of the polymerase chain reaction means DNA contamination can ruin an entire experiment. Tidiness and adherence to a strict set of protocols can avoid disaster.

THE polymerase chain reaction (PCR)[1-3] is a powerful, exquisitely sensitive[3,4] technique with applications in many fields such as molecular biology, medical diagnostics, population genetics and forensic analysis. The use of specific DNA amplification, as reflected by the number of publications reporting the use of PCR, has indeed grown "exponentially" in recent months. We and others[5] are concerned, however, that some investigators may not be using adequate care in experimental design and execution when using PCR to detect only a few molecules of a target DNA sequence. A false positive or mistyping may occur when the majority of molecules to be detected arise from exogenous sources rather than from the sample itself.

Obviously, the fewer molecules one is trying to detect, the more one should guard against this possibility. The use of PCR for sensitive detection is complicated by the fact that the product of the amplification serves as the substrate for the generation of more product. A single PCR cycle produces very large numbers of amplifiable molecules that can potentially contaminate subsequent amplifications of the same target sequence. This kind of contamination has been termed PCR product "carryover" to differentiate it from contamination by naturally arising DNA (Fig. 1).

An analogy is useful to illustrate the scale of the contamination problem when using PCR to detect very few molecules. A typical PCR reaction can generate 10^{12} molecules of amplified DNA in a 0.1 ml reaction[3]. Imagine the uniform dilution of this number of molecules in a volume of liquid that fills an Olympic-sized swimming pool (~50 m × 25 m × 2 m). A 0.1 ml aliquot of liquid from this pool would contain 400 amplifiable molecules.

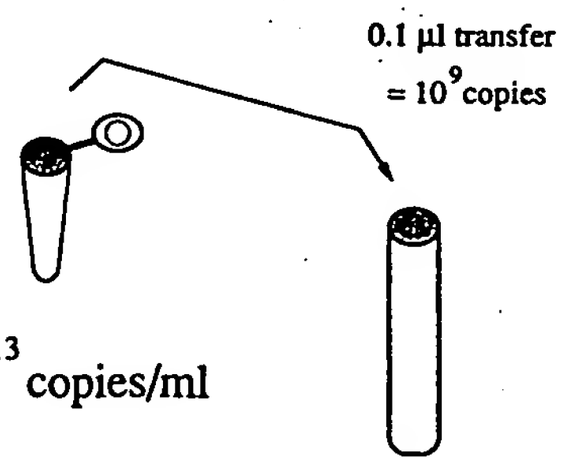

FIG. 1 The high concentration of amplifiable DNA in a completed PCR (typically ~ 10^{13} per ml) makes the inadvertent transfer of even a small volume to an unamplified sample quite serious. Reusing a pipette tip transfers 0.1 µl — roughly 10^9 copies of sequence. A microgram of human DNA contains only 1.4 × 10^5 copies of a single-copy gene.

To control contamination, one must prevent the physical transfer of DNA between amplified samples, and between positive and negative experimental controls. The following precautions have dramatically reduced false positive rates in our laboratories that perform PCR detection of HIV sequences[6] (in which as few as 15 copies of DNA per sample are routinely detected). Those who use PCR for less demanding procedures, such as the preparation of DNA fragments from plasmid DNA templates, may be able to adapt a less stringent form of these guidelines with successful results.

Tips for better PCR

Physically isolate PCR preparations and products. To prevent carryover of amplified DNA sequences, we prepare samples in a separate room or biosafety hood from that in which the reactions are performed. The UV germicidal lamps in most biosafety hoods quickly damage any DNA left on exposed surfaces, making it unsuitable for subsequent amplification (G. Sensabaugh, personal communication), and further eliminating the possibility of contamination between samples. Separate sets of supplies and pipetting devices are dedicated for sample preparation and for setting up reactions.

Autoclave solutions. We routinely autoclave deionized water and those buffer solutions used in PCR and sample preparation that can be autoclaved without affecting their performance. Autoclaving under conditions that provide bacterial decontamination degrades DNA to a very low molecular weight (N. Arnheim, personal communication). We also autoclave disposable pipette tips and microcentrifuge tubes. Primers, dNTPs and *Taq* DNA polymerase cannot be autoclaved.

Aliquot reagents. We divide reagents into aliquots to minimize the number of repeated samplings necessary. All reagents used in the PCR are prepared, divided and stored in an area that is free of PCR-amplified product. Similarly, oligonucleotides used for amplification are synthesized and purified in a PCR product-free environment. It is advisable to record the lot(s) of reagents used so that if contamination occurs, it can more easily be traced.

Use disposable gloves. Our researchers wear gloves and change them frequently, at the least when entering or reentering the isolation area. Changing gloves reduces the possibility of the transfer of amplifiable DNA from outside the isolation area and between samples.

Avoid splashes. Some types of sample tube have caps that require so much force to remove that liquid at the bottom of the tube may be splashed out. We use caps that do not require that much effort, and change gloves if such a splash occurs. It is also a good idea to spin any liquid down from the sides and top of the closed tube before attempting to open it.

Use positive displacement pipettes. The barrel of pipetting devices may become contaminated with aerosols containing sample DNA, leading to cross-contamination of samples. To prevent this, we use positive displacement pipettes with disposable tips and plungers.

"Premix" reagents. When possible, we mix reagents before dividing them into aliquots. All PCR reagents can be combined into a "premixture" which can then be pipetted into reaction vessels containing DNA. This minimizes the number of sample transfers and the chances for sporadic contamination. When dispensing the mixture, we pipette a "no DNA" negative control last so that it reflects the total reagent handled.

Add DNA last. Non-sample components, such as mineral oil, pre-mixed dNTPs, primers, buffer and enzyme, should be added to the amplification vessels before sample DNA. This minimizes cross-contamination by reducing the number of opportunities for the inadvertent transfer of DNA. After the addition of DNA, we cap each tube before proceeding to the next sample.

Choose positive and negative controls carefully. We do not use a highly concentrated solution of plasmid DNA containing the target sequence as our positive control, because it would introduce as many amplifiable molecules into the sample preparation area as a typical PCR. If plasmid DNA containing the target sequence is used as a positive control, it should be diluted substantially. Depending upon the detection system, as few as 100 copies of target may suffice as a positive control.

We include "no DNA" reagent controls and negative sample controls with each set of amplifications. The reagent controls should contain all the necessary components for PCR, except template DNA.

Negative sample controls should not contain target sequences, but should have gone through all the sample preparation steps. Choosing negative controls for our HIV studies was complicated by the fact that PCR enabled HIV sequences to be detected from samples which were negative by all other tests. In the end, we used samples from low-risk individuals with well-known histories.

Avoiding other pitfalls

The amount of PCR product generated is sometimes insufficient, requiring re-amplification after enrichment by gel electrophoresis. If the amount of amplified target sequence DNA in a particular gel slice is very low, one should be wary of cross-contamination from analogous PCR products or plasmid DNA containing the target sequence run in other lanes of the gel. To prevent carryover from equipment used in previous experiments, gel apparatus and combs should be soaked in 1 M HCl to depurinate any residual DNA, new razor blades should be used to excise each gel band, and the surface of the UV transilluminator should be covered with a fresh sheet of plastic wrap for each gel. Other potential sources of contamination include purified restriction fragments of target sequence, dot-blot apparatus, microtome blades, centrifuges, centrifugal vacuum devices and dry ice or ethanol baths. Most of these items are also amenable to treatment, if necessary, with 1 M HCl.

A final caution

If there is any doubt at all about a critical result, it is best to repeat the experiment again. The negative controls described above can rule out reagent contamination, but cannot guarantee against sporadic contamination events. Fortunately, the odds of a sporadic contamination event occurring twice the same way are very low. The net error rate of a series of tests is also usually lower than that of a single trial, even if the sporadic error rate of the repeated test is higher. PCR is simple and rapid, and consumes so little of most samples that repeat experiments can be performed, even in forensic work[7].

Kits for performing PCR are available from Perkin-Elmer Cetus under the trademark GeneAmp. □

Shirley Kwok and Russell Higuchi are at the Departments of Infectious Diseases and Human Genetics, Cetus Corporation, Emeryville, California 94608, USA. For more information, fill in reader service number 100.

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BioExpo on show

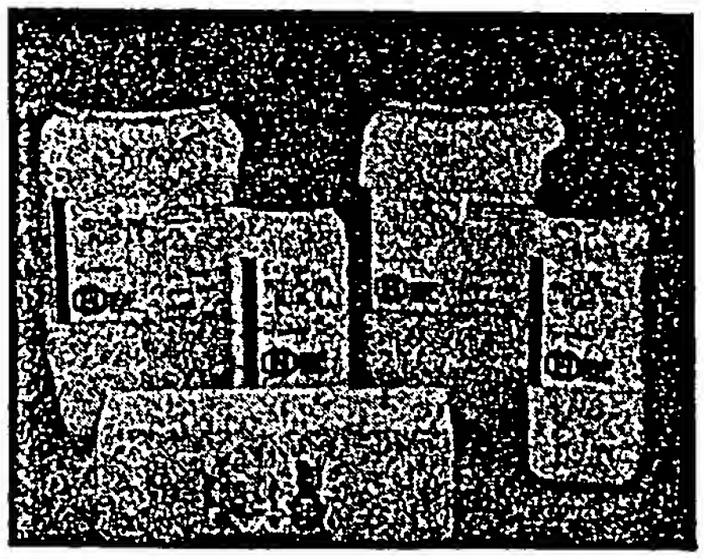
At next week's BioExpo in Paris, France, new products range from an automated loop inoculation device to a crystallized Tris buffer preparation.

THE MAbTrap G from Pharmacia LKB is designed for the **purification of monoclonal and polyclonal IgG** from ascites fluid, serum and cell culture supernatants (*Reader Service No. 101*). Because Protein G binds only IgG and its subclasses, separating out other immunoglobulins, Pharmacia LKB says the MAbTrap G offers high capacity, high flow rate and high recovery.

Tired of laboriously flaming loops when streaking petri plates? The Swiss company Tecnomara has an **automatic flame inoculation** system, dubbed the Rondoflame (*Reader Service No. 102*). The base unit of the system is an automatic bunsen burner fireboy that can hold four inoculating loops inserted into specialized holders on a rotation assembly at 90° angles to the plane of rotation. One loop is always in the optimal position for flaming: the fireboy burns only as long as necessary to flame the loop and automatically advances the loop to the take-up position. The device can even be programmed to rotate clockwise or counterclockwise for left- and right-handed use. The Rondoflame is marketed at a cost of $1,350 (US).

Boehringer Mannheim is now offering its Ultrapure **Tris buffer in crystalline form** (*Reader Service No. 103*). The company says the buffer dissolves easier in solution than powdered buffers without clumping, and that it is free of contaminating proteases, DNases and RNases. At a purity of over 99.9 per cent, BMB says the Tris buffer is totally clear in solution, low in metal content and costs $160 (US) for 5 kg.

A new range of supports for **ion exchange chromatography** is being added to the product list at IBF Biotechnics (*Reader Service No. 104*). The Trisacryl Plus supports, based on the Trisacryl matrix, are of fundamental benefit, says IBF Biotechnics: they are resistant to extremes in pH, high flow rates, freezing, autoclaving and bacterial contamination. The Trisacryl Plus range is available in 300-ml bottles for the M grade (40–80 μm bead size) for $118 (US) and in 1-litre bottles for the LS grade (80–160 μm) for $355 (US).



Trisacryl Plus chromatography media.

Beckman Instruments has a new series of **HPLC autosamplers** that operate on-line as part of the company's System Gold series of liquid chromatographs or stand alone as part of another system (*Reader Service No. 105*). The model 507 autosampler is designed for laboratories that analyse large sample volumes on a daily basis. Operating features include a four-quadrant, refrigerated sample holder with a 96-vial capacity and a separate vial for rinse liquid. Vial access is direct, and column temperature control is available. The $10,500-16,225 (US) model 507 also offers variable injection volumes, column switching, and pre-column reagent addition, mixing and reaction. □

These notes are compiled by Cary Prince from information provided by the manufacturers. To obtain additional information about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.

RESEARCH ARTICLE

Enzymatic Amplification of β-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia

Randall K. Saiki, Stephen Scharf, Fred Faloona, Kary B. Mullis
Glenn T. Horn, Henry A. Erlich, Norman Arnheim

Abstract. *Two new methods were used to establish a rapid and highly sensitive prenatal diagnostic test for sickle cell anemia. The first involves the primer-mediated enzymatic amplification of specific β-globin target sequences in genomic DNA, resulting in the exponential increase (220,000 times) of target DNA copies. In the second technique, the presence of the β^A and β^S alleles is determined by restriction endonuclease digestion of an end-labeled oligonucleotide probe hybridized in solution to the amplified β-globin sequences. The β-globin genotype can be determined in less than 1 day on samples containing significantly less than 1 microgram of genomic DNA.*

Recent advances in recombinant DNA technology have made possible the molecular analysis and prenatal diagnosis of several human genetic diseases. Fetal DNA obtained by aminocentesis or chorionic villus sampling can be analyzed by restriction enzyme digestion, with subsequent electrophoresis, Southern transfer, and specific hybridization to cloned gene or oligonucleotide probes. With polymorphic DNA markers linked genetically to a specific disease locus, segregation analysis must be carried out with restriction fragment length polymorphisms (RFLP's) found to be informative by examining DNA from family members (*1*, *2*).

Many of the hemoglobinopathies, however, can be detected by more direct methods in which analysis of the fetus alone is sufficient for diagnosis. For example, the diagnosis of hydrops fetalis (homozygous α-thalassemia) can be made by documenting the absence of any α-globin genes by hybridization with an α-globin probe (*3*–*5*). Homozygosity for certain β-thalassemia alleles can be determined in Southern transfer experiments by using oligonucleotide probes that form stable duplexes with the normal β-globin gene sequence but form unstable hybrids with specific mutants (*6*, *7*).

Sickle cell anemia can also be diagnosed by direct analysis of fetal DNA. This disease results from homozygosity of the sickle-cell allele (β^S) at the β-globin gene locus. The S allele differs from the wild-type allele (β^A) by substitution of an A in the wild-type to a T at the second position of the sixth codon of the β chain gene, resulting in the replacement of a glutamic acid by a valine in the expressed protein. For the prenatal diagnosis of sickle cell anemia, DNA obtained by amniocentesis or chorionic villus sampling can be treated with a restriction endonuclease (for example, Dde I and Mst II) that recognizes a sequence altered by the β^S mutation (*8*–*11*). This generates β^A- and β^S-specific restriction fragments that can be resolved by Southern transfer and hybridization with a β-globin probe.

We have developed a procedure for the detection of the sickle cell mutation that is very rapid and is at least two orders of magnitude more sensitive than standard Southern blotting. There are two special features to this protocol. The first is a method for amplifying specific β-globin DNA sequences with the use of oligonucleotide primers and DNA polymerase (*12*). The second is the analysis of the β-globin genotype by solution hybridization of the amplified DNA with a specific oligonucleotide probe and subsequent digestion with a restriction endonuclease (*13*). These two techniques increase the speed and sensitivity, and lessen the complexity of prenatal diagnosis for sickle cell anemia; they may also be generally applicable to the diagnosis of other genetic diseases and in the use of DNA probes for infectious disease diagnosis.

Sequence amplification by polymerase chain reaction. We use a two-step procedure for determining the β-globin genotype of human genomic DNA samples. First, a small portion of the β-globin gene sequence spanning the polymorphic Dde I restriction site diagnostic of the β^A allele is amplified. Next, the presence or absence of the Dde I restriction site in the amplified DNA sample is determined by solution hybridization with an end-labeled complementary oligomer followed by restriction endonuclease digestion, electrophoresis, and autoradiography.

The β-globin gene segment was amplified by the polymerase chain reaction (PCR) procedure of Mullis and Faloona (*12*) in which we used two 20-base oligonucleotide primers that flank the region to be amplified. One primer, PC04, is complementary to the (+)-strand and the other, PC03, is complementary to the (−)-strand (Fig. 1). The annealing of PC04 to the (+)-strand of denatured genomic DNA followed by extension with the Klenow fragment of *Escherichia coli* DNA polymerase I and deoxynucleotide triphosphates results in the synthesis of a (−)-strand fragment containing the target sequence. At the same time, a similar reaction occurs with PC03, creating a new (+)-strand. Since these newly synthesized DNA strands are themselves template for the PCR primers, repeated cycles of denaturation, primer annealing, and extension result in the exponential accumulation of the 110–base pair region defined by the primers.

An example of the degree of specific gene amplification achieved by the PCR method is shown in Fig. 2A. Samples of DNA (1 μg) were amplified for 20 cycles and a fraction of each sample, equivalent to 36 ng of the original DNA, was subjected to alkaline gel electrophoresis and transferred to a nylon filter. The filter was then hybridized with a ^{32}P-labeled 40-base oligonucleotide probe, RS06, which is complementary to the target sequence (Fig. 1A) but not to the PCR primers. The results, after a 2-hour autoradiographic exposure, show that a fragment hybridizing with the RS06 probe

The authors are in the Department of Human Genetics, Cetus Corporation, 1400 Fifty-Third Street, Emeryville, California 94608. The present address for N.A. is Department of Biological Sciences, University of Southern California, Los Angeles 90089-0371.

migrates at the position expected of the amplified target DNA segment (110 bases) (lanes 1 and 2). No hybridization with the RS06 probe could be detected in unamplified DNA (lane 4). When PCR amplification was performed on a DNA sample derived from an individual with hereditary persistence of fetal hemoglobin in which both the δ- and β-globin genes are deleted (*14*), again no 110-base fragment was detected (lane 3). To estimate the yield and efficiency of 20 cycles of PCR amplification, we prepared a Southern blot that contained 36 ng of an amplified genomic DNA sample and a dilution series consisting of various amounts of cloned β-globin sequence. The efficiency was calculated according to the formula: $(1 \div X)^n = Y$, where X is the mean efficiency per cycle, n is the number of PCR cycles, and Y is the extent of amplification (yield) after n cycles (for example, a 200,000-fold increase after 20 cycles). The amounts of cloned β-globin sequences used in this experiment were calculated to represent efficiencies of 70 to 100 percent.

The reconstructions were prepared by digesting the β-globin plasmid, pBR328::β^A, with the restriction enzymes Hae III and Mae I. Both of these enzymes cleave the β-globin gene within or very near to the 20 base regions that hybridize to the PCR primers, generating a 103–base pair (bp) fragment that is almost identical in size and composition to the 110-bp segment created by PCR amplification. After hybridization with the RS06 probe and autoradiography, the amplified genomic sample was compared with the known standards, and the result indicated an overall efficiency of approximately 85 percent (Fig. 2B), representing an amplification of about 220,000 times (1.85^{20}).

Distinguishing the β^A and β^S alleles by the oligomer restriction method. We have previously described a rapid solution hybridization method that can indicate whether a genomic DNA sample contains a specific restriction enzyme site at, in principle, any chromosomal location (*13*). This method, called oligomer restriction (OR), involves the stringent hybridization of a ^{32}P end-labeled oligonucleotide probe to the specific segment of the denatured genomic DNA which spans the target restriction site. The ability of a mismatch within the restriction site to prevent cleavage of the duplex formed between the probe and the target genomic sequence is the basis for detecting allelic variants. The presence of the restriction site in the target DNA is revealed by the appearance of a specific

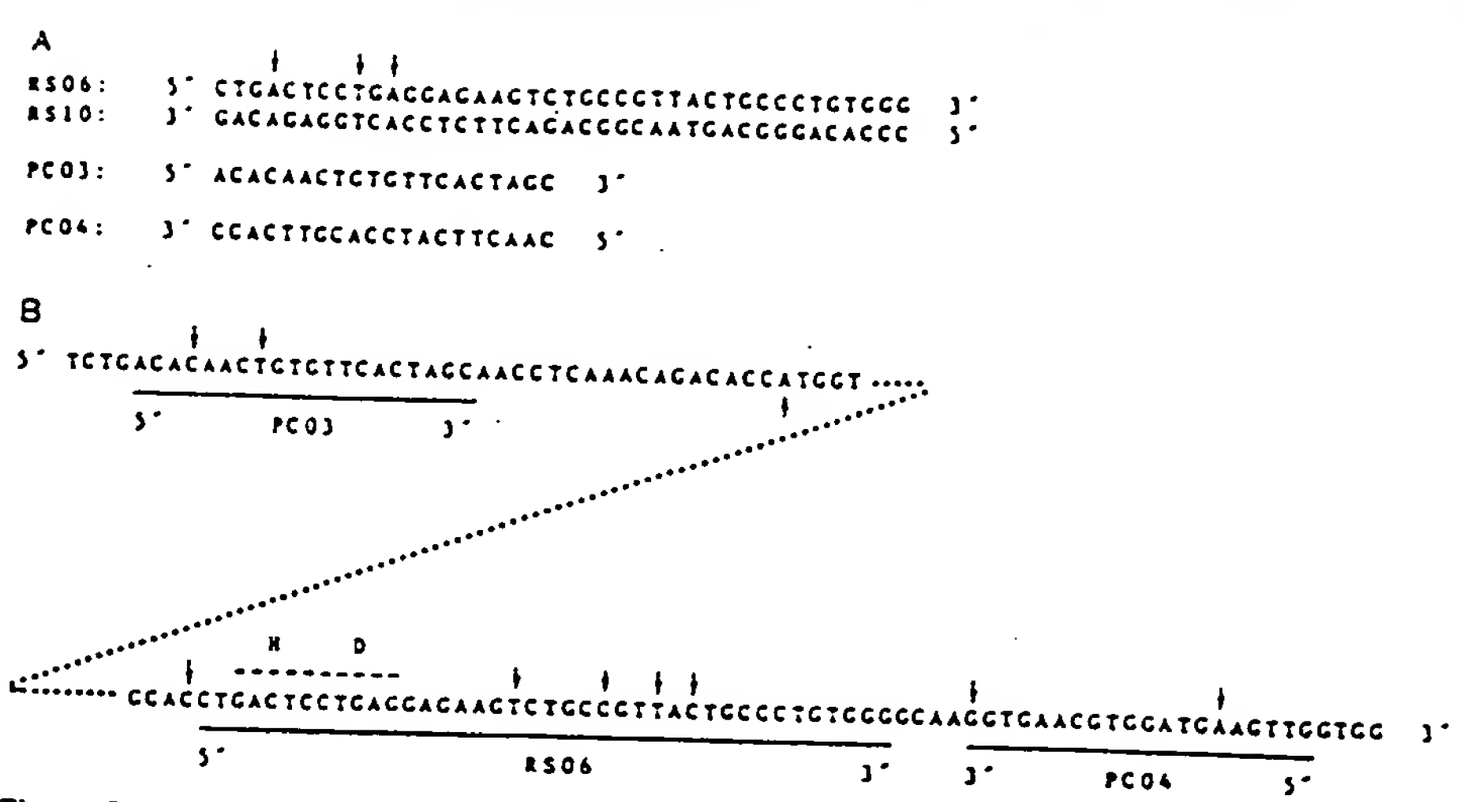


Fig. 1. Sequence of synthetic oligonucleotide primers and probe and their relation to the target β-globin region. (A) The primer PC03 is complementary to the (−)-strand and the primer PC04 is complementary to the (+)-strand of the β-globin gene. The probe RS06 is complementary to the (−)-strand of the wild-type (β^A) sequence of β-globin. RS10 is the "blocking oligomer", an oligomer complementary to the RS06 probe except for three nucleotides, indicated by the downward arrows. It is added before enzyme digestion to the OR reaction to anneal to the excess RS06 oligomer and prevent nonspecific cleavage products due to hybridization of RS06 to nontarget DNA (*13*). Because of the mismatches within the Dde I and Hinf I restriction sites, the RS06/RS10 duplex is not cleaved by Dde I and Hinf I digestion. (B) The relation between the primers, the probe, and the target β-globin sequence. The upward arrow indicates the β-globin initiation codon. The downward arrows indicate nucleotide differences between β- and δ-globin. The polymorphic Dde I site (CTCAG) is represented by a single horizontal dashed line (D), and the invariant Hinf I (GACTC) site is represented by double horizontal dashed lines (H).

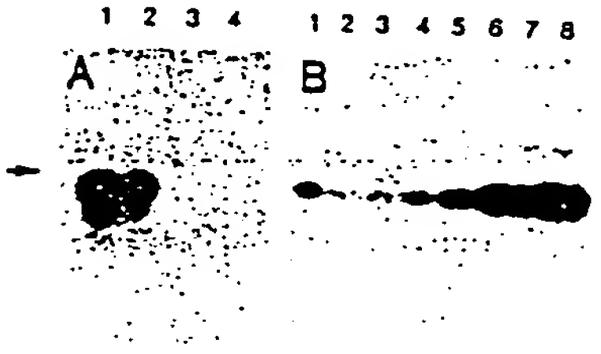


Fig. 2. Southern analysis of PCR amplified genomic DNA with the RS06 probe. (A) Samples (1 μg) of genomic DNA were dispensed in microcentrifuge tubes and adjusted to 100 μl in a buffer containing 10 m*M* tris, *p*H 7.5, 50 m*M* NaCl, 10 m*M* $MgCl_2$, 1.5 m*M* deoxynucleotide trisphosphate [(dNTP) each of all four was used], 1 μ*M* PC03, and 1 μ*M* PC04. After heating for 5 minutes at 95°C (to denature the genomic DNA), the tubes were centrifuged for 10 seconds in a microcentrifuge to remove the condensation. The samples were immediately transferred to a 30°C heat block for 2 minutes to allow the PC03 and PC04 primers to anneal to their target sequences. At the end of this period, 2 μl of the Klenow fragment of *E. coli* DNA polymerase I (Biolabs, 0.5 unit/μl in 10 m*M* tris, *p*H 7.5, 50 m*M* NaCl, 10 m*M* $MgCl_2$) was added, and the incubation was allowed to proceed for an additional 2 minutes at 30°C. This cycle—denaturation, centrifugation, hybridization, and extension—was repeated 19 more times, except that subsequent denaturations were done for 2 instead of 5 minutes. (The final volume after 20 cycles was 140 μl.) Thirty-six nanograms of the amplified genomic DNA (5 μl) were applied to a 4 percent Nusieve (FMC) alkaline agarose minigel and subjected to electrophoresis (50 V), for 2 hours until the bromcresol green dye front reached 4 cm. After neutralization and transfer to Genetrans nylon membrane (Plasco), the filter was "prehybridized" in 10 ml 3× SSPE (1× SSPE is 0.18*M* NaCl, 10 m*M* NaH_2PO_4, 1 m*M* EDTA, *p*H 7.4), 5× DET (1× DET is 0.02 percent each polyvinylpyrrolidone, Ficoll, and bovine serum albumin; 0.2 m*M* tris, 0.2 m*M* EDTA, *p*H 8.0), 0.5 percent SDS, and 30 percent formamide for 4 hours at 42°C. Hybridization with 1.0 pmol of phosphorylated (with [γ-^{32}P]ATP) RS06 (~5 μCi/pmol) in 10 ml of the same buffer was carried out for 18 hours at 42°C. The filter was washed twice in 2× SSPE, 0.1 percent sodium dodecyl sulfate (SDS) at room temperature for 30 minutes, and autoradiographed at −70°C for 2 hours with a single intensification screen. (Lanes 1 to 3) DNA's isolated from the cell lines Molt4, SC01, and GM2064, respectively. Molt4 is homozygous for the normal, wild-type allele of β-globin ($\beta^A\beta^A$), SC-1 is homozygous for the sickle cell allele ($\beta^S\beta^S$), and GM2064 is a cell line in which the β- and δ-globin genes have been deleted ($\Delta\Delta$) (*13*). (Lane 4) Contains 36 ng of Molt4 DNA that was not PCR amplified. The horizontal arrow indicates the position of a 114-base marker fragment obtained by digestion of pBR328 with Nar I. (B) Thirty-six nanograms of 20-cycle amplified Molt4 DNA (see above) was loaded onto a Nusieve gel along with measured amounts of Hae III–Mae I digested pBR328::β^A (*13*) calculated to represent the molar increase in β-globin target sequences at PCR efficiencies of 70, 75, 80, 85, 90, 95, and 100 percent (lanes 2 to 8, respectively). DNA was transferred to Genetrans and hybridized with the labeled RS06 probe as described above. (Lane 1) Molt4 DNA (36 ng); (lanes 2 to 8) 7.3×10^{-4} pmol, 1.3×10^{-3} pmol, 2.3×10^{-3} pmol, 4.0×10^{-3} pmol, 6.8×10^{-3} pmol, 1.1×10^{-2} pmol, and 1.9×10^{-2} pmol of pBR328::β^A, respectively (*20*).

labeled fragment generated by cleavage of the probe.

For the diagnosis of sickle cell anemia, the probe was designed to be complementary to a region of the β-globin gene locus surrounding the sixth codon. In the β^A allele, the nucleotide (nt) sequence at this position contains a Dde I restriction site, but due to the single base mutation, this site is absent in the β^S allele. Our strategy for generating specific probe cleavage products for each allele is shown in Fig. 3. It is based on the presence of an invariant Hinf I restriction enzyme site immediately adjacent to the polymorphic Dde I restriction site. Resolution of the labeled oligomer cleavage products produced by sequential digestion with Dde I and Hinf I allows us to distinguish between the two alleles. In an individual homozygous for the wild-type β-globin allele AA, Dde I digestion will produce a labeled octamer (8 nt) from the probe. Because of its short length, the 8-nt cleavage product will dissociate from the genomic target DNA and the subsequent digestion with Hinf I has no effect. In the case of SS homozygotes, however, Dde I digestion does not cleave the probe since a base pair mismatch exists in the recognition sequence formed between the probe and target DNA. The invariant Hinf I site will then be cleaved during Hinf I digestion, releasing a labeled trimer (3 nt). In an AS heterozygote, both a trimer and an octamer would be detected. The resolution of the intact 40-base probe, the 8-nt and the 3-nt cleavage products is achieved by polyacrylamide gel electrophoresis. Experiments testing the sequential digestion strategy with plasmids carrying the β^A and β^S alleles show that, in each case, the expected probe cleavage products were produced (Fig. 4).

Analysis of genomic DNA samples by PCR and OR. Eleven DNA samples derived from lymphoblastoid cell lines or white blood cells were analyzed for their β-globin genotype by standard Southern blotting and hybridization of the Mst II RFLP (*10*), identifying the genotypes of the samples as either AA, AS, or SS. Six of these samples (and one additional one) were then amplified by PCR for 20 cycles starting with 1 μg of DNA each. An aliquot of the amplified DNA sample (one-fourteenth of the original 1-μg sample) was hybridized to the RS06 probe and digested with Dde I and then Hinf I. A portion (one-tenth) of this oligomer restriction reaction was analyzed on a polyacrylamide gel to resolve the cleavage products, and the results obtained after 6 hours of autoradiography are shown Fig. 5. The high sensitivity achieved with the PCR and OR method is demonstrated by the strength of the autoradiographic signal derived from only 1/140 of the original 1-μg sample (7 ng). Each sample determined to be AA by RFLP analysis showed a strong 8-nt fragment while those typed as SS showed a strong 3-nt fragment. Analysis of the known AS samples revealed both cleavage products.

In the analysis of the AA samples, a faint 3-nt could be detected in addition to the primary 8-nt signal. The reasons for this band remain unclear, although incomplete Dde I cleavage or the occasional failure of the 8-nt fragment to disassociate from the target DNA may contribute to the nonspecific 3-nt product generated by Hinf I digestion. In the analysis of the SS samples, a very faint 8-nt band was also observed in addition to the expected 3-nt signal. We have determined that the background 8-nt product detected in SS samples can be attributed to the δ-globin gene, which is highly homologous to β-globin. The nucleotide sequence of the two β-globin primers used for amplification is shown in Fig. 1. The downward pointing arrows indicate the differences between the β- and δ-globin genes. We hypothesized that the faint 8-nt signal observed in the SS samples was due to some amplification of the δ-globin gene by these primers and the subsequent cross-hybridization of the amplified δ sequences with the RS06 probe used in the OR procedure. δ-Globin has the same Dde I site as normal β-globin, and the duplex formed between an amplified δ gene segment and the RS06 probe would be expected to yield an 8-nt fragment on Dde I digestion even

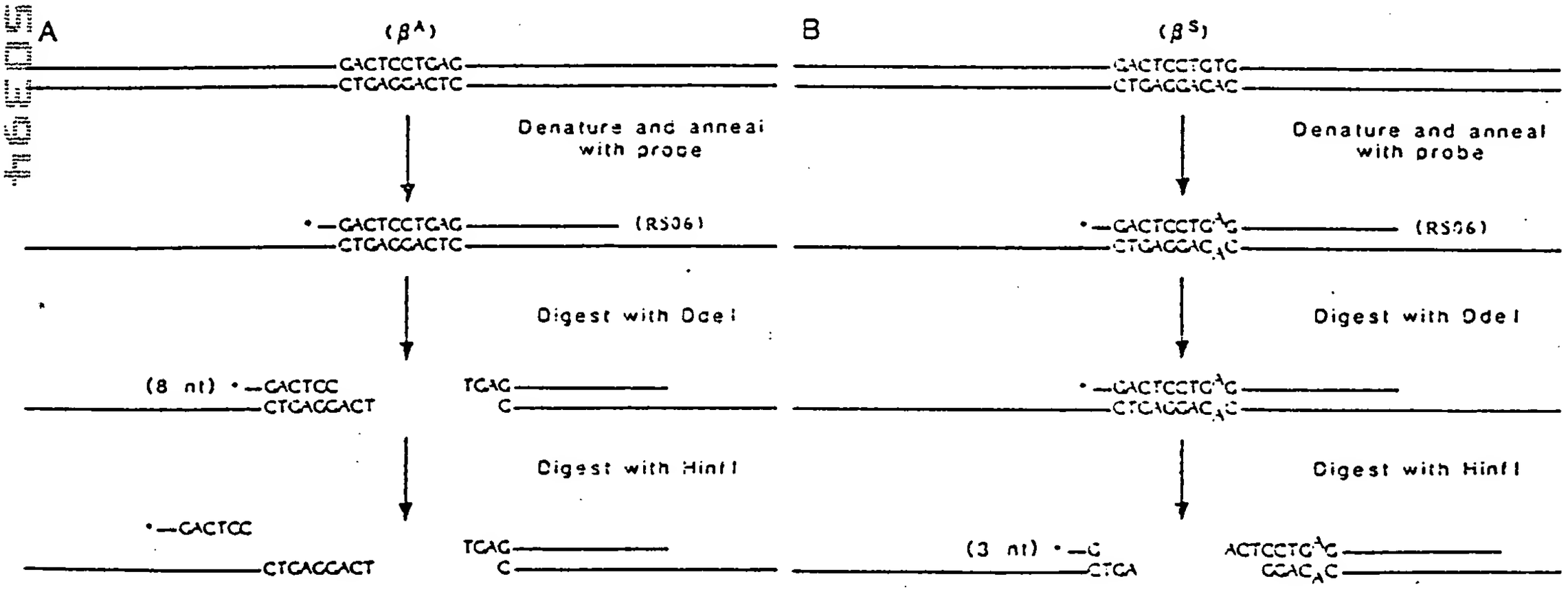


Fig. 3. Schematic diagram of oligomer restriction by sequential digestion to identify β^A- and β^S-specific cleavage products. The DNA sequences shown are the regions of the β-globin genomic DNA and the RS06 hybridization probe containing the invariant Hinf I site (GANTC, where N represents any nucleotide) and the polymorphic Dde I site (CTNAG). The remaining DNA sequences are represented as solid horizontal lines. The asterisk indicates the position of the radioactive ^{32}P label attached to the 5′-end of the RS06 probe with polynucleotide kinase. (A) Outline of the procedure and expected results when RS06 anneals to the normal β-globin gene (β^A). After denaturation of the genomic DNA and hybridization of the labeled RS06 probe to the complementary target sequence in the β^A gene, digestion of the probe-target hybrid with Dde I causes the release of a labeled (8-nt) cleavage product. Because of the relatively stringent conditions during Dde I digestion, the 8-nt cleavage product dissociates from the genomic DNA and the subsequent digestion with Hinf I has no effect. (B) Outline of Dde I and Hinf I digestion after hybridization of the RS06 probe to the sickle cell allele (β^S). As a consequence of the β^S mutation, the probe-target hybrid contains an A-A mismatch within the Dde I site and is not cleaved by the Dde I endonuclease. The Hinf I site, however, remains intact and digestion with that enzyme generates a labeled 3-nt product. Thus, the presence of the β^A allele is revealed by the release of a labeled 8-nt fragment, while the presence of β^S is indicated by a labeled 3-nt fragment.

though there are sequence differences (four mismatch out of 40 bases) between RS06 and δ-globin. It is likely that δ-globin sequences may be amplified to some extent and detected weakly with the RS06 probe in all DNA samples, but that its contribution to the total signal is very small and detectable only when the sample is SS and no 8-nt fragment from the β-globin gene is expected. We tested this hypothesis by treating an SS DNA sample before amplification with the enzyme Mbo I. Since there is a recognition site for this enzyme in the target DNA of the δ- but not the β-globin gene, cleavage of the δ gene between the regions that hybridize to the PCR primers should prevent its subsequent amplification (but not of β-globin). Our results showed that an SS DNA sample, first digested with Mbo I, gave only the 3-nt product but not the 8-nt product, this is consistent with the hypothesis of δ-globin amplification.

Effect of PCR cycle number on detection threshold. The strength of the autoradiograph signal detected by OR as a function of PCR cycle number and autoradiographic exposure was examined. The signal intensity after 20 cycles is at least 20 times as strong as that for 15 cycles and the determination of the β-globin genotype can be made with an autoradiographic exposure for only 2 hours (Fig. 6). The observed increase of ≥20-fold is consistent with our estimates of 85 percent efficiency per cycle, calculated from the data in Fig. 2B ($1.85^5 = 21.7$). Coupled with the time that it takes to actually carry out the PCR and OR procedures, a 20-cycle PCR allows a diagnosis to be made in less than 10 hours with a DNA sample of 1 μg.

Since all of the previous PCR experiments were done with 1 μg of genomic DNA, we explored the effect of using significantly smaller amounts of DNA as template for PCR amplification. The results obtained with 20 cycles of PCR amplification on 500, 100, 20, and 4 ng of DNA from an AS individual are shown in Fig. 7. After analysis of 1/40 of each sample by the OR procedure and a 20-hour autoradiographic exposure, the β-globin genotype could be easily determined on DNA samples of 20 ng or about 100 times less than is needed for a typical Southern transfer and hybridization experiment. In this experiment, only a small fraction (1/40) of the starting material was placed on the gel; therefore it should be possible to analyze samples of less than 20 ng of genomic DNA (20 ng is equivalent to approximately 6000 haploid genomes) if a larger proportion of the material was utilized in the OR and gel electrophoresis steps.

Diagnostic applications of the PCR-OR system. When currently available methods are used, the completion of a prenatal diagnosis for sickle cell anemia takes a period of several days after the DNA is isolated. With 1 μg of genomic DNA, the β-globin genotype can be determined by the PCR-OR method in less than 10 hours: 20 cycles of amplification requires about 2 hours (each full cycle takes 6 to 7 minutes in our protocol), the oligomer restriction procedure involving liquid hybridization and enzyme digestions require an additional 2 hours, and the electrophoresis takes about an hour. Autoradiographic exposure for 4 hours is sufficient to generate a strong signal. Because this method includes a liquid hybridization protocol and involves the serial addition of reagents to a single tube, it is simpler to perform than the standard Southern transfer and hybridization procedure. Prior to electrophoresis, all of the reactions can be done in two small microcentrifuge tubes and could readily be automated.

The sensitivity, as well as the speed and simplicity, of this procedure is also important for clinical applications. Twenty nanograms of starting material can provide an easily detectable result in an overnight autoradiographic exposure. This sensitivity makes the PCR-OR method particularly valuable in cases

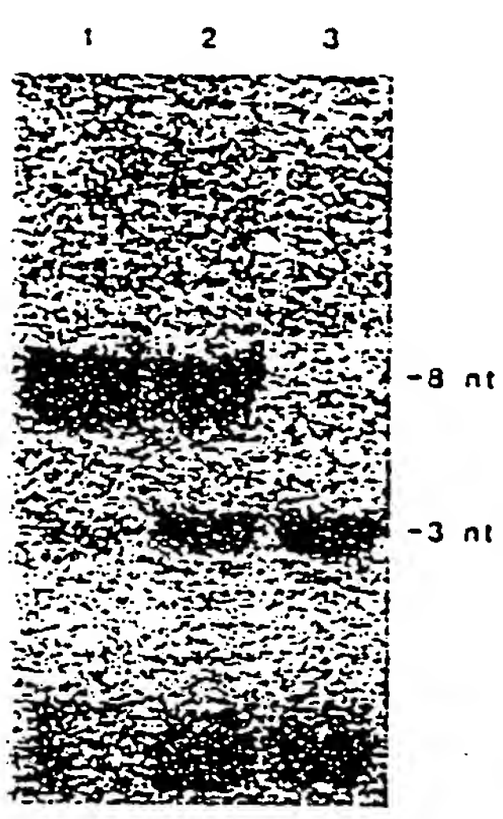
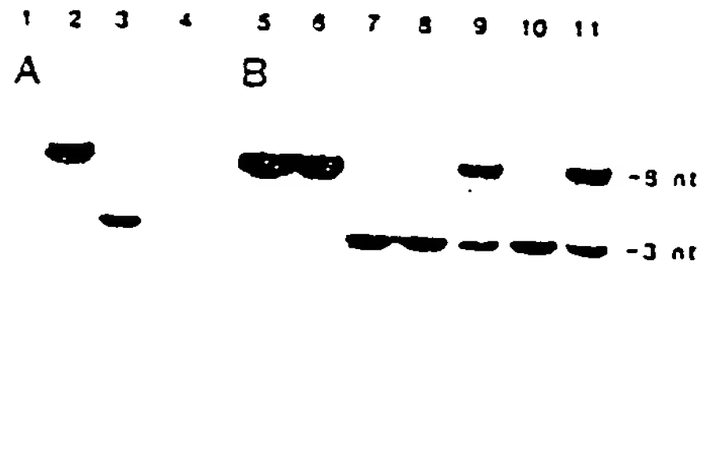


Fig. 4. Demonstration of OR sequential digestion with cloned β-globin genes. The sequential digestion strategy was demonstrated by annealing the RS06 probe to the β-globin plasmids pBR328::β^A and pBR328::β^S (*13*). The methods were similar to those described (*13*). Cloned β-globin DNA (45 ng; 0.01 pmol) was placed in a microcentrifuge tube, adjusted to 30 μl with TE buffer (10 m*M* tris, 0.1 m*M* EDTA, *p*H 8.0), overlaid with 0.1 ml of mineral oil. The DNA was denatured by heating for 5 to 10 minutes at 95°C. Ten microliters of 0.6*M* NaCl containing 0.02 pmol of phosphorylated (with [γ-^{32}P]ATP) RS06 probe oligomer (~5 μCi/pmol) was added and annealed for 60 minutes at 56°C. Unlabeled RS10 blocking oligomer (4 μl; 200 pmol/ml) (Fig. 1) (*13*) was then added, and the hybridization was continued for 5 to 10 minutes. Next, 5 μl of 100 m*M* MgCl$_2$ and 1 μl of Dde I (Biolabs, 10 units) was added and incubated for 20 minutes at 56°C; 1 μl of Hinf I (Biolabs, 10 units) was added and digestion was continued for 20 minutes at the same temperature. The reaction was terminated by the addition of 4 μl of 100 m*M* EDTA and 6 μl of tracking dye to a final volume of 61 μl; a portion (8 μl) (6 ng, 0.0013 pmol) was applied to a 0.75-mm thick, 30 percent polyacrylamide minigel (19 acrylamide:1 bis) in a Hoefer SE200 apparatus and subjected to electrophoresis (300 V) for 1 hour until the bromphenol blue dye front reached 3 cm. The top 1.5 cm of the gel, containing intact RS06, was cut off and discarded. The remaining gel was autoradiographed with a single intensification screen for 13 hours at −70°C. (Lane 1) six nanograms of pBR328::β^A; (lane 2) 3 ng of pBR328::β^A plus 3 ng of pBR328::β^S; and (lane 3) 6 ng of pBR328::β^S.



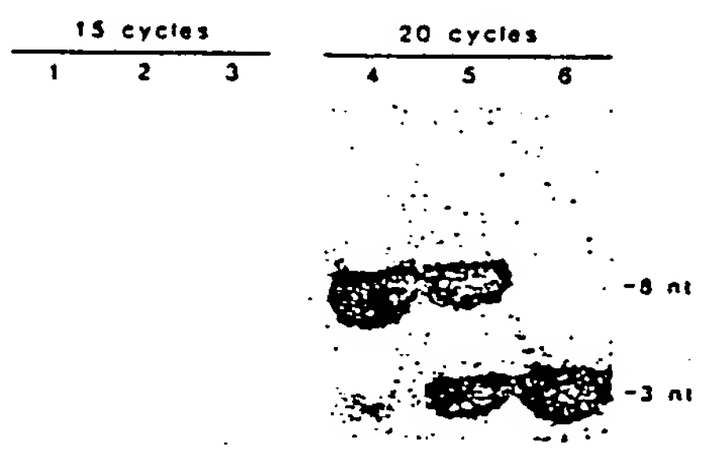


Fig. 5 (left). Determination of the β-globin genotype in human genomic DNA with PCR-OR. Samples (1 μg) of human genomic DNA were amplified for 20 cycles (as described in Fig. 2A). The amplified DNA's (71 ng) were hybridized to the RS06 probe and serially digested with Dde I and Hinf I (as described in Fig. 4). Each sample (6 μl) was analyzed by 30 percent polyacrylamide gel electrophoresis and autoradiographed for 6 hours at −70°C with one intensification screen. Each lane contains 7 ng of genomic DNA. (Lane 1) Unamplified Molt4 DNA (negative control); (lane 2) amplified Molt4 ($\beta^A\beta^A$); (lane 3) SC-1 ($\beta^S\beta^S$); (lane 4) GM2064 (2.2); (lanes 5 to 11) clinical samples CH1 ($\beta^A\beta^A$), CH2 ($\beta^A\beta^A$), CH3 ($\beta^S\beta^S$), CH4 ($\beta^S\beta^S$), CH7 ($\beta^A\beta^S$), CH8 ($\beta^S\beta^S$), and CH12 ($\beta^A\beta^S$), respectively. Fig. 6 (right). Effect of cycle number on signal strength. Genomic DNA (1 μg) from the clinical samples CH2 ($\beta^A\beta^A$), CH12 ($\beta^A\beta^S$), and CH5 ($\beta^S\beta^S$) were amplified for 15 and 20 cycles and equivalent amounts of genomic DNA (80 ng) were analyzed by oligomer restriction. (Lanes 1 to 3) DNA (20 ng) from CH2, CH12, and CH5, respectively, amplified for 15 cycles; (lanes 4 to 6) DNA (20 ng) from CH2, CH12, and CH5, respectively, amplified for 20 cycles. Autoradiographic exposure was for 2.5 hours at −70°C with one intensification screen.

where poor DNA yields are obtained from prenatal samples. In addition, DNA samples of poor quality (very low average molecular weight) can give excellent results in the PCR-OR protocol.

The PCR method is likely to be generally applicable for specific gene amplification since a fragment encoding a portion of the HLA-DQα locus has recently been amplified with this procedure (*15*). We have carried out PCR amplification on a 110-bp β-globin sequence with an overall efficiency per cycle of about 85 percent. We have also amplified longer β-globin fragments (up to 267 bp), but the yield was lower under our standard conditions. Efficient amplification of a 267-bp fragment required some variation in the PCR procedure. In principle, increasing the number of PCR cycles should yield even greater amplification than that reported here (~220,000-fold after 20 cycles).

Our method for the diagnosis of sickle cell anemia involves the coupling of the PCR procedure with that of oligomer restriction. It was designed to distinguish between two alleles that differ by a polymorphic restriction site. The PCR-OR method is applicable as well to the diagnosis of other diseases where the lesion directly affects a restriction enzyme site or where the polymorphic site is in strong linkage disequilibrium with the disease-causing locus. If the polymorphism is in linkage equilibrium with the disease, PCR-OR requires family studies to follow the inheritance of the disease locus.

In the case of the β-globin locus, the presence of the invariant Hinf I restriction site adjacent to the polymorphic Dde I site allows a sequential digestion procedure to identify both the β^A and β^S alleles. In principle, this approach does not require that the two sites be immediately adjacent but only that the cleavage product generated by digestion at the polymorphic site dissociate from the target to prevent cutting at the invariant site. Since the restriction enzyme digestion conditions used here are fairly stringent for hybridization, we estimate that the polymorphic and invariant sites could perhaps be separated by as much as 20 bp.

The application of the PCR method to prenatal diagnosis does not necessarily depend on a polymorphic restriction site or on the use of radioactive probes. In fact, the significant amplification of target sequences achieved by the PCR method allows the use of nonisotopically labeled probes (*16*). Amplified target sequences could also be analyzed by a number of other procedures including those involving the hybridization of small labeled oligomers which will form stable duplexes only if perfectly matched (*6*, *7*, *17*, *18*) and the recently reported method based on the electrophoretic shifts of duplexes with base pair mismatches (*19*). The ability of the PCR procedure to amplify a target DNA segment in genomic DNA raises the possibility that its use may extend beyond that of prenatal diagnosis to other areas of molecular biology.

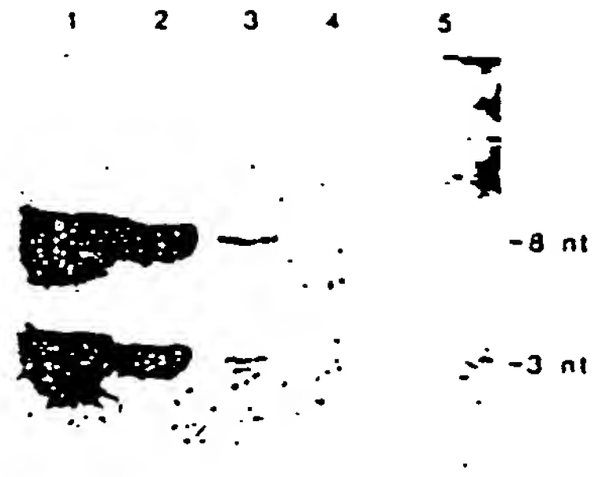


Fig. 7. Detection threshold for PCR-OR. Fivefold serial dilutions of genomic DNA (500, 100, 20, and 4 ng) from the clinical sample CH12 ($\beta^A\beta^S$) were amplifed by 20 cycles of PCR and one-tenth each reaction (50, 10, 2, and 0.4 ng) was analyzed by OR. The gel continued (lane 1) genomic DNA (12.5 ng); (lane 2) 2.5 ng; (lane 3) 0.5 ng; (lane 4) 0.1 ng; (lane 5) 12.5 ng genomic DNA from the globin deletion cell line GM2064. Autoradiographic exposure was for 20 hours at −70°C with an intensification screen.

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20. Calculation of the amounts of pBR328:β^A^ needed as standards to estimate PCR efficiency was done in the following way. If we assume that a human haploid genome size is 3×10^9 bp, 36 ng of DNA is equivalent to 1.8×10^{-4} pmol. The extent of amplification after 20 cycles at, for example, 85 percent efficiency is obtained with $(1.8 \times 10^{-4})(1.85^{20})$ or 4.0×10^{-3} pmol of plasmid DNA (Fig. 2B, lane 5).

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Selective enrichment of specific DNA, cDNA and RNA sequences using biotinylated probes, avidin and copper-chelate agarose

Andrew A.Welcher[1], Anthony R.Torres[2] and David C.Ward[1,3]*

Departments of [1]Molecular Biophysics and Biochemistry, [2]Laboratory Medicine and [3]Human Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA

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ABSTRACT

We have developed a general procedure for the rapid and efficient enrichment of specific DNA, RNA or cDNA sequences. Biotinylated DNA or RNA is used as a hybridization probe in solution, avidin is then added to label both the probe and hybrid molecules, and the hybridization mixture chromatographed over cupric-iminodiacetic acid agarose beads. Avidin-probe and avidin-hybrid molecules are selectively retained on the column; non-hybridized sequences are contained in the flow-through fraction. Sequences retained on the column are recovered in high yield by the addition of ethylenediamine tetracetic acid in the buffer. The method can be used in both subtractive enrichment and positive selection protocols. Here we report its application to the isolation of Neisseria gonorrhoeae specific genomic DNA clones and the purification of a cDNA subpopulation representing mRNA sequences that are over-expressed in murine Friend cells after dimethylsulfoxide induction.

INTRODUCTION

The preparation of recombinant DNA clones containing specific DNA or cDNA sequences is often time consuming, requiring either a major effort to isolate the appropriate mRNA or extensive screening of large plasmid, phage or cosmid libraries with nucleic acid or antibody probes. The availability of rapid and efficient methods for preenriching target nucleic acids prior to cloning, particularly if they are of unknown sequence or molecular complexity, can significantly reduce the time and effort of the overall process. Several positive selection procedures have been described for the enrichment of target sequences for which specific hybridization probes exist; most of these involve hybridization to, and elution from, probe sequences immobilized on a solid support, such as agarose, dextran, cellulose or nitrocellulose (reviewed in 1). These procedures generally work well, although the hybridization efficiency is often low due to polynucleotide immobilization on the solid matrix.

Preferential enrichment of specific mRNAs has been achieved, in the absence of nucleotide or amino acid sequence information, when an antibody to

the translation product of the mRNA is available. Positive selection by immunoprecipitation of nacent RNA on polysomes (2) has resulted in the extensive purification of several mRNAs that are relatively rare (3,4), however, size selection of mRNA, based on the immunoprecipitation of in vitro translation products (5), gives a more modest enrichment. In the absence of either specific sequence data or antibody reagents few preenrichment strategies exist. However, when cell populations which differentially express a mRNA of interest are available, cDNA can be made from mRNA of the "producer" cell, hybridized to an excess of mRNA from the "non-producer" cell, and the double-stranded hybrids, reflecting shared sequences, separated from free cDNAs by chromatography on hydroxylapatite [HAP] (6). The efficiency of this subtractive enrichment can be improved further by hybridizing the enriched cDNA in the flow-through fraction to an excess of "producer" mRNA and recovering the resultant hybrids from HAP (7). cDNA clones specific for interferon induced genes (8) murine histocompatibility complex genes (9) Aspergillus differention genes (10) and α_{2u} globulin (11) have been obtained by screening libraries with HAP-selected cDNAs. Nevertheless, since fractionation on HAP is based on the extent of doublestrandedness, RNAs with extensive secondary structure can heavily contaminate the hybrid fraction and reduce the selectivity.

In this paper we describe a method that can be used for both positive and subtractive sequence enrichment which exploits the high specificity and avidity of the interaction between biotin and avidin (12) or Streptavidin (13) and a metal chelate adsorbent (14) that reversibly binds these protein-biotinylated nucleic acid complexes. We also report the isolation of a set of Neisseria gonorrhoeae specific DNA clones by screening a genomic library with DNA obtained after subtractive hybridization with biotinylated Neisseria meningitidis DNA, which shares ~ 80% sequence homology with N. gonorrhoeae (15). cDNAs complementary to mRNA sequences overexpressed in dimethylsulfoxide treated Friend leukemia cells were also prepared by subtractive hybridization with photobiotinylated RNA from uninduced cells.

MATERIALS AND METHODS

Bacterial Isolates

Neisseria meningitidis (ATCC 13090, group B), Neisseria gonorrhoeae (ATCC E 27630), Escherichia coli RC109, and clinical isolates from the Clinical Microbiology Laboratory at Yale-New Haven Hospital, were used in the experiments reported here. The N. gonorrhoeae strain was shown (M. Li,

personal communications) to be free of the high copy number plasmid that is present in 95% of N. gonorrhoeae isolates (16). Neisseria cultures were propagated on chocolate agar plates.

DNA Isolation

The Marmur method for DNA isolation (17) was used with minor modifications. Neisseria organisms were scraped with a sterile cotton swab from 8 chocolate agar plates (10 cm in diameter) after overnight growth, and suspended in 20 mls of lysis buffer (0.15 M NaCl-10 mM EDTA-1% SDS). E. coli was grown in YT broth, concentrated by centrifugation and suspended in 20 ml of lysis buffer. After lysis at 65°C for 30 minutes, 3.5 mls of 6 M sodium perchlorate was added. The solution was extracted with 12.5 mls chloroform-isoamyl alcohol (24:1) in a 50 ml polypropylene centrifuge tube placed on a shaker platform for 30 minutes. The aqueous and organic phases were separated by centrifugation at 12,000 RPM for 10 minutes, using a SS34 rotor in a Sorvall centrifuge. The aqueous layer was removed and the extraction procedure repeated. The DNA was precipitated overnight with 2.5 volumes of absolute ethanol at -20°C, pelleted by centrifugation at 10,000 RPM for 10 minutes, and then resuspended in 0.5 mls of 10 mM Tris-HCl, pH 7.5 - 1mM EDTA (T.E.). Ribonuclease A (Cooper Biomedical) was added to a final concentrations of 100 µg/ml and the solution incubated at 37°C for 1 hr. Proteinase K (EM Biochemicals) and sodium dodecylsulfate (BDH Chemical) were then added at concentrations of 200 µg/ml and 0.2% w/v, respectively, and the solution incubated a further 2 hrs at 37°C. The sample was extracted twice with phenol and once with ethyl ether. Sodium chloride was added to a final concentration of 0.2 M, and the DNA ethanol precipitated as before. The DNA was resuspended in 100 µl of T.E. and its concentration measured spectrophotometrically. If the 260/280 ratio was below 1.6, the Proteinase K digestion and phenol extraction steps were repeated a second time.

DNA Digestion

Purified bacterial DNAs were digested with restriction endonucleases (New England Biolabs) using buffers and incubation conditions recommended by the manufacturer. Routinely, 100 µg of N. meningitidis DNA was digested with 50 U of AluI in a volume of 200 µl at 37°C for 4 hours, while 10 µg of N. gonorrhoeae DNA was digested with 10 U Sau3A I in a volume of 100 µl at 37°C for 4 hours. The DNAs were extracted once with phenol, once with ether, ethanol precipitated and pelleted as before, and then resuspended in 50 µl of T.E.

DNA Labeling

N. meningitidis DNA, digested with AluI, was labeled with ^{3}H dATP (NEN)

and Bio-11-dUTP (18) by nick-translation (19). Four reactions each containing 10 µg of N. meningitidis were nick-translated with 30 U DNA Polymerase I (NEB), DNase I (Sigma) at a final concentration of 20 pg/ml, in a volume of 200 µl, at 14°C for one hour. The four reactions were pooled, EDTA was added to a concentration of 10 mM, and the DNA was concentrated by centrifugal lyophilization (Savant speed vac) to a volume of approximately 200 µl. Unincorporated nucleotides were removed from the solution by chromatography on a 3 ml Sephadex G-50 (Pharmacia) column equilibrated in T.E.

The N. gonorrhoeae DNA was labeled with ^{32}P dCTP (Amersham) by filling in the Sau3A I restriction sites with DNA Polymerase I Klenow fragment (NEB). The reaction mixture (80 µl), containing 1 µg N. gonorrhoeae (digested with Sau3A I), 250 µCi ^{32}P dCTP (specific activity ∿ 3000 Ci/mmole), 20 U Klenow enzyme, dGTP, dATP, and dTTP, each at a concentration of 100 µM, was incubated at room temperature for 15 minutes. The DNA was phenol extracted two times, extracted once with ether, and then chromatographed through a 1 ml G-50 spin column (21) equilibrated in T.E. The specific activity of the N. gonorrhoeae DNA was $5x10^7$ cpm/µg.

Growth and Induction of Erythroleukemic Cells

The murine Friend cell line MEL 745 was grown in Dulbecco's Modification of Eagle's Medium (Flow) supplemented with 13% (V/V) heat inactivated fetal calf serum (Gibco), glutamine 2 mM, penicillin 102 U/ml, and streptomycin 75 U/ml. Cells were propagated at 37°C in an atmosphere containing 5% CO_2, with the cell density being maintained between $1x10^5$ and $1x10^6$ cells per ml. Erythroid differentiation was induced by the addition of dimethylsulfoxide (Aldrich) to a final concentration of 1.8% for a period of 72 hrs. The extent of β-globin induction was monitored by the benzidine color assay (20).

Preparation of poly(A)$^+$ RNA and cDNA Synthesis

Cytoplasmic RNA was prepared from induced and uninduced MEL 745 cells and poly(A)$^+$ RNA was purified by chromatography on oligo(dT)-cellulose (Collaborative Research) as described by Maniatis et al. (21) . First strand cDNA synthesis was carried out in a reaction volume of 50 µl, containing 50 mM Tris-HCl, pH 8.1, 5 mM $MgCl_2$, 2 mM DTT, 40 mM KCl, 100 µM dGTP, 100 µM dTTP, 100 µM dATP, 50 µM dCTP, 150 µCi of α ^{32}P dCTP (∿ 3000 Ci/mmole), 50 µg of oligo (dT_8) (Pharmacia), 0.5 µg poly(A)$^+$ RNA from DMSO induced cells, 2.5 µg Actinomycin D, and 100 units of AMV reverse transcriptase (Life Sciences), for 1 hour at 41°C. RNA was removed by treating the reaction with NaOH at a final concentration of 0.2 M for 1 hr at 41°C. EDTA was then added to 20 mM, and unincorporated nucleotides and excess primer removed by chromatography over a

1.5 ml G-50 Sephadex column. The cDNA in the void volume was then extracted once with phenol and concentrated by butanol extraction to a volume of 100 µl. The cDNA was then extracted with ether one time, ethanol precipitated and the cDNA resuspended in 5 µl of distilled water. The specific activity of the cDNA pool was $9x10^7$ cpm/µg.

Labeling of RNA with Photobiotin

Non-induced poly(A)$^+$ RNA (40 µg in 40 µl of T.E.) was mixed with 40 µg of photobiotin (Vector Labs) dissolved in 40 µl dH_2O for a total volume of 80 µl. The solution, in a 1.5 ml Eppendorf tube, was placed on ice and irradiated for 20 minutes by a sunlamp (GE model RSM, 275W) at a height of 10 cm. After adjusting the solution to 0.1 M Tris-HCl - 1 mM EDTA, pH 8.0, it was extracted twice with an equal volume of 1-butanol. Sodium acetate, pH 5.2, was added to the aqueous phase at a final concentration of 0.25 M and the RNA precipitated with 2.5 volumes of 100% ethanol. After 30 min at -20°C, the RNA was pelleted by centrifugation at 4°C for 10 minutes in a Microfuge (Beckman) at 13,000 RPM. The RNA was washed with 70% ethanol, repelleted as above, and then resuspended in 40 µl of T.E.

Solution Hybridization with Biotinylated DNA

Ten µg of N. meningitidis DNA (labeled with bio-11-dUTP and ^{3}H-dCTP) was added to 1 µg N. gonorrhoeae DNA (labeled with ^{32}P-dCTP) in a 1.5 ml Eppendorf tube. The mixture was concentrated by centrifugal lyophilization to a volume of approximately 100 µl, and desalted on a 1 ml G-50 spin column equilibrated in T.E., pH 7.5. The solution was concentrated as above, to a small volume (10-20 µl). Sodium chloride and sodium phosphate (pH 6.8) were added to a final concentration of 0.75 M and 0.05 M, respectively. The solution was heat denatured at 100°C for 4 minutes, quick chilled on ice, overlaid with paraffin oil (J.T. Baker Chemical Co.), and placed in a heating block set at 55°C. The hybridization was allowed to proceed for 15 hrs for a Cot of 40.

At the end of the hybridization, the paraffin oil was removed with a Pasteur pipet and the volume adjusted up to 90 µl with freshly prepared 20 mM $NaHCO_3$-1 M NaCl (pH 7.7), 60 µl of 5 M NaCl was added, followed by 150 µl of a 1 mg/ml solution of Avidin-DN (Vector labs) in 0.05 M $NaHCO_3$ pH 8.2, - 0.1% thimerosal. The tube was gently mixed then placed in a heating block set at 55°C until the sample was applied to the chelate column.

Chromatography on Cupric-iminodiacetic Acid-Agarose

A silanized 1 ml disposable syringe (Becton-Dickinson) plugged with silanized glass wool was packed with 0.4 mls of iminodiacetic acid-agarose (Pierce Chemical). The column was washed with ten column volumes of dH_2O,

then 0.2 mls of a $CuSo_4$ solution (5 mg/ml in dH_2O) was passed through the column; the copper charged column becomes pale blue. The column was washed with ten column volumes dH_2O, then ten column volumes 20 mM $NaHCO_3$-1 M NaCl (pH 7.7). At this point, 300 µl of the bicarbonate solution was added to the DNA-avidin solution (see above), the sample gently mixed, and the solution was slowly passed over the column. Fractions were collected and the column was washed with the bicarbonate solution until the radioactivity of the eluate was reduced to background levels. Bound DNA was eluted by washing the column with 50 mM EDTA-1 M NaCl-0.1% SDS. As the copper ion was released, the column turned from blue to white; the DNA was routinely found in the first drop or two of blue elution buffer. The distribution of N. gonorrhoeae DNA in the unbound and bound fractions was quantitated by Cerenkov counting or with a Beckman Model 170 flow-through radioisotope detector.

Preparing DNA for Subsequent Hybridization Cycle

Unbound DNA fractions were pooled and concentrated by butanol to a volume of approximately 0.5 mls. The solution was dialyzed overnight at 4°C against one liter of T.E. pH 7.5-10 mM NaCl, concentrated by butanol to approximately 100 µl, and then extracted once with ether. The selection procedure was then continued by adding 10 µg of labeled N. meningitidis DNA and repeating the hybridization and chromatographic steps described before. Four or five hybridization cycles were done in the experiments reported here.

Dot Blots

N. gonorrhoeae, N. meningitidis, and E. coli DNAs (300 ng of each) were immobilized on nitrocellulose (22) and dot blot-hybridization used to monitor the enrichment of N. gonorrhoeae DNA fragments. The nitrocellulose membranes were prehybridized for two hours at 42°C in 50% formamide, 5XSSC, 25 mM $NaPO_4$ pH 6.8, 5 x Denhardt's, 250 µg/ml sonicated salmon sperm DNA. 2 ng/ml of selected ^{32}P-labeled N. gonorrhoeae DNA and 10% dextran sulfate were added to the prehybridization cocktail and the solution hybridized for 16 hours at 42°C. The membranes were washed twice for 3 minutes in 2XSSC-0.1% SDS, once for 3 minutes in 0.2XSSC-0.1% SDS, twice for 15 minutes each in 0.1XSSC-0.1% SDS at 55°C, then 1 time for 3 minutes in 2XSSC-0.1% SDS. The membranes were air dried, and placed under X-ray film (Kodak XAR) with a DuPont Cronex Lightning intensifying screen for 4 to 15 hours at -70°C.

Colony Hybridization

N. gonorrhoeae DNA digested with Sau3AI, was shotgun cloned into the Bam HI site of pUC-13 (P.L.-Pharmacia) and the DNA used to transform the bacterial strain JM103. Transformants were plated out on YT-Amp-XGal plates, and white

colonies (containing inserts) were streaked with a toothpick onto YT grid plates, with 100 colonies per plate. Replica grids were prepared on nitrocellulose filters as previously described (21).

The selected DNA from the fifth hybridization cycle, extensively enriched in N. gonorrhoeae sequences, was used as a probe to screen the colony bank. Hybridization conditions were the same as for the dot blots; the washing procedures were also the same with two modifications. The washes with 0.1XSSC-0.1% SDS were done at 60°C instead of at 55°C, and the washes were done for a total of 45 minutes instead of 30 minutes to reduce the background signal. Washed filters were air dried, and placed under XAR-5 film with an intensifying screen for 40 hrs at -70°C.

Isolation of N. gonorrhoeae Specific Colonies

Colonies which hybridized to the affinity selected N. gonorrhoeae DNA were picked by toothpick and grown in 2 mls of YT-broth. Mini-plasmid preparations were done as described (21). Plasmid DNA was digested with EcoRI and Xbal, subjected to electrophoresis on a 1.4% agarose gel, run in a buffer of 40 mM Tris-HCl, pH 8.5 - 5 mM Na acetate-1 mM EDTA, at 100 volts for 6 hrs. The DNA was visualized by EtBr staining, inserts were cut out of the gel, and the DNA was electroeluted from the agarose slices (21). The DNA was concentrated with butanol, ether extracted, then desalted on a 1 ml G-50 spin column equilibrated in T.E. DNA was then nick-translated (19). ^{32}P-labeled DNA was used as a hybridization probe for the dot-blots, as described before, to test if the inserts were specific for N. gonorrhoeae. Random inserts from the same library were tested in the same way to determine the percentage of N. gonorrhoeae specific clones.

Solution Hybridization with Biotinylated RNA

Ten µg of non-induced RNA (labeled with biotin) was added to 50 ng of ^{32}P-labeled cDNA made from induced RNA. The final solution hybridization mixture contained 0.65 M NaCl, 0.04 M $NaPO_4$ pH 6.8, 1 mM EDTA, and 0.05% SDS in a total volume of 20 µl. The RNA was denatured by heating at 100°C for 3 minutes, quick cooled on ice, then covered with mineral oil. Hybridization was done at 60°C for 24 hrs. From the nucleotide complexity of Erythroleukemia cell mRNAs (6), these conditions should give a Rot of 20. After hybridization, the mineral oil was removed, 10 µl of 5 M NaCl and 75 µl of a 1 mg/ml solution of Avidin-DN (Vector Labs) added, and the mixture was incubated for 30 minutes at 60°C. The solution was then passed through a copper chelate column (0.3 ml) with the initial column eluate being recycled through the column twice before initiating the washing steps. Subsequent

chromatographic steps and dot blot analysis of cDNA enrichment were done as described for the N. gonorrhoeae DNA selection experiments.

RESULTS

Separation of Biotinylated and Non-biotinylated Nucleic Acids on Metal Chelate Columns

Metal chelate chromatography (14) has been used previously to fractionate cationic proteins which exhibit differential affinities for heavy metal ions (23,24). Exposed histidine, tryptophan and cysteine residues form relatively stable complexes with immobilized copper or zinc ions in neutral aqueous solutions, however, these complexes are readily reversed by the addition of EDTA or by lowering the pH. We reasoned that metal chelate columns could provide an effective, and inexpensive, means of separating biotinylated nucleic acid species from their non-biotinylated counterparts if avidin, streptavidin or antibiotin antibodies could be used as bridging proteins between the column and the biotinylated molecules. The data shown in Figure 1

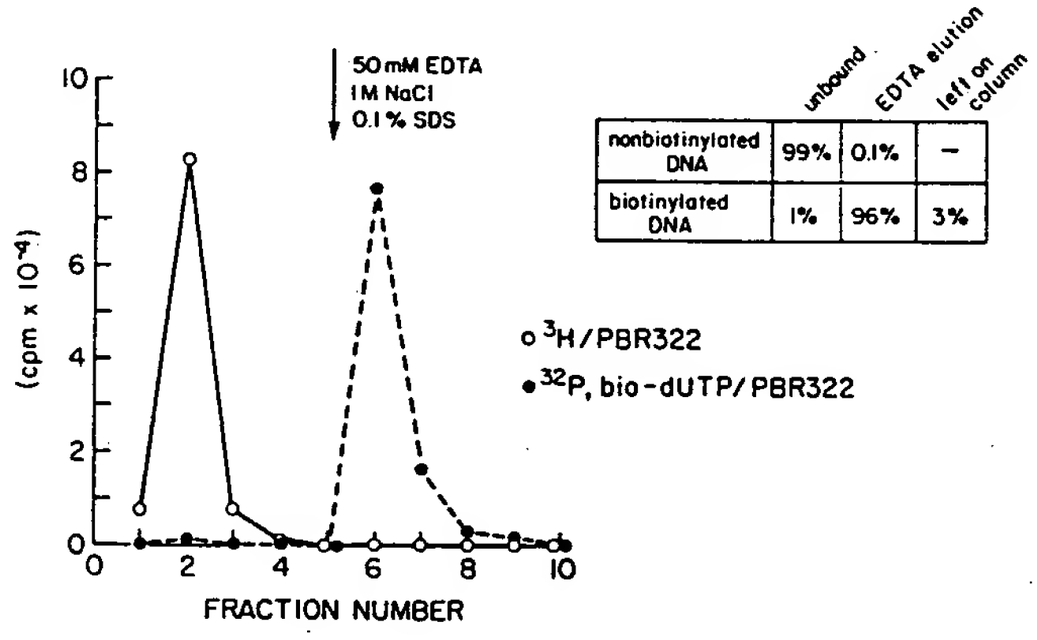


Figure 1. Separation of biotinylated DNA from non-biotinylated DNA by chromatography on copper-chelate agarose. Two samples of pBR322 DNA were nick-translated, one being labeled with ^{3}H-dCMP (o), the other with ^{32}P-dCMP and Bio-11-dUMP (●), each at a specific activity of 10^{7} cpm/µg. To 10 ng of each DNA in 50 µl of T.E. was added 50 µl of 2 M NaCl and 100 µl of Avidin-DN (1 mg/ml). The solutions were kept on ice for 5 min and then chromatographed over 0.2 ml columns of iminodiacetic acid agarose precharged with 0.11 ml of $CuSo_4$ (5 mg/ml). 0.2 ml fractions were collected and counted in hydrofluor. Columns were washed with 1 ml of 20 mM $NaHCO_3$-1 M NaCl and then the column bound DNA was eluted with 50 mM EDTA-1 M NaCl-0.1% SDS. The inset shows the distribution of the two DNA samples during the chromatography.

demonstrates that this is indeed the case. Two samples of pBR322 DNA were nick-translated, one labeled with Bio-11-dUTP and ^{32}P-dCTP, the other labeled with ^{3}H-dCTP in the absence of Bio-11-dUTP. Both samples were incubated with an excess of avidin-DN for 5 minutes and then chromatographed over iminodiacetic acid-agarose charged with copper sulfate (see Methods). Greater than 99% of the ^{3}H-pBR322 DNA was found in the flow-through fraction while ~ 99% of the ^{32}P-biotinylated pBR322 DNA was retained on the column under identical conditions. Retention of the biotinylated DNA was absolutely dependent on the prior addition of avidin; the chromatographic profile of the biotinylated DNA (Bio-DNA) was identical to that of the control DNA without added avidin. The column-bound Bio-DNA was recovered with high efficiency (~ 96%) by washing the adsorbent with a solution of 50 mM EDTA-1 M NaCl-0.1% SDS. In contrast, the EDTA-eluate from the control DNA column contained 0.1% or less of the input counts.

In some experiments a small fraction, here 3%, of the Bio-DNA remained on the column even after extensive washing with elution buffer. This residual material appears to be high molecular weight DNA-avidin intermolecular aggregates that are physically trapped on the gel matrix. Since avidin is a tetrameric protein capable of binding 4 molecules of biotin simultaneously, it can, particularly at low avidin-DNA ratios, generate Bio-DNA - Bio-DNA crosslinks. By using a judicious avidin excess (10-fold or more relative to Bio-dNMP incorporated) when forming the DNA-protein complexes in solution, this non-specific entrappment can be maintained at low levels (0.5-3%). However, if necessary, this DNA subset can be recovered by incubating the column in proteinase K and pronase or by incubating at elevated temperatures (50-60°C) in the presence of 1-2% SDS.

The copper-chelate agarose has a high binding capacity for avidin and associated biotinylated molecules. Titration studies demonstrated that several different commercial lots of iminodiacetic acid-agarose bound 15-20 mg of avidin per ml of resin when charged with copper ions; this binding occurs rapidly, virtually instantaneously, between pH 6 and 8 and is not affected by NaCl concentrations as high as 2 molar. Efficient fractionation of Bio-DNA from non-biotinylated DNA also was achieved with streptavidin as the bridging protein or when the chelate was charged with zinc ions (data not shown). Bio-DNA, however, does not bind as tightly to the Zn-chelate, as suggested by prior experiments on protein chromatography (14). Metal chelate agarose also yielded excellent results on both micro (ng) and bulk (multi mg) scales.

We next tested the specificity and efficiency of the copper-chelate

agarose in selecting hybrid molecules formed during solution hybridization reactions with biotinylated probes. ^{32}P-labeled pBR322 DNA was prepared as before and hybridized in solution with a five-fold molar excess of pBR322 DNA, linearized by EcoRI digestion and labeled with ^{3}H-dAMP and Bio-11-dUMP using a DNA polymerase "fill-in" reaction. Following hybridizaion, the mixture was divided into three parts, one part was chromatographed over avidin-agarose (Sigma), a second chromatographed over avidin-DN-agarose (Vector Labs) while the third was incubated with avidin-DN in solution for 5 min and then chromatographed over copper-chelate-agarose. All three chromatographic methods resulted in the retention of > 90% of the biotinylated probe (containing only 4 biotin residues per pBR322 DNA molecule) in a single column passage. However, only 18% and 21% of the ^{32}P-labeled target molecules were retained on the two avidin-agarose resins while 48% was recovered on the copper-chelate column. Similar results were obtained in a comparison of the ability of the copper-chelate agarose and streptavidin-agarose (Bethesda Research Labs) to bind RNA-cDNA hybrids formed with an RNA probe labeled with photobiotin (25). Although both columns retained > 95% of the input probe, 54% of the target cDNA was retained on the copper-chelate column while only 15% was bound to the streptavidin-agarose. Efficient recovery of hybrid molecules on avidin-agarose or streptavidin-agarose required more extended column incubation periods or multiple cycles through the affinity matrix. These observations suggest the formation of avidin-biotin or streptavidin-biotin nucleic acid complexes is appreciably more efficient in solution than when either protein is immobilized on a solid matrix. The results further demonstrate that the capture efficiency of such complexes on copperchelates is quite high.

A similar comparison of the binding of ^{32}P-labeled, biotin-free, DNA to these various affinity matrices indicated that the copper-chelate column exhibited the lowest level of interaction with non-biotinylated DNA; by washing extensively with 20 mM $NaHCO_3$-1 M NaCl the non-specific binding can easily be reduced to less than 1 part in 10^4. In contrast, the non-specific sticking of "control" DNA to avidin-agarose or streptavidin-agarose was generally in the range of 0.1-0.5%.

Enrichment of N. gonorrhoeae DNA by Subtractive Hybridization

As a first test of the utility of this fractionation procedure for selective sequence enrichment, we set out to isolate a family of Neisseria gonorrhoeae genomic DNA clones containing sequences which were absent from the closely related organism Neisseria meningitidis. These two human pathogens,

causitive agents of gonorrhoeae and acute bacterial meningitis, respectively, have approximately 80% sequence homology (15). The basic subtractive hybridization strategy we used is outlined below.

The DNA of interest (N. gonorrhoeae) first was digested with the restriction enzyme Sau3A. This enzyme gives DNA fragments between 0.5 and 5 kb with an average size of ~ 2 kb, thus maximizing the amount of potential N. gonorrhoeae specific sequences to be obtained during the selection procedure, and it yields fragments which, after reannealing, could be cloned directly into a BamHI site of an appropriate plasmid vector. Alternatively, the DNA was ^{32}P-labeled by "filling-in" the single-stranded ends of the Sau3A fragments with DNA polymerase and ^{32}P-dCTP. The N. meningitidis (don't-want) DNA was cut with a second restriction enzyme, AluI, and labeled with Bio-11-dUTP by nick-translation. Both steps minimize the risk of cloning any meningitidis DNA which might escape subsequent fractionation procedures. A 10-fold excess of the biotinylated meningitidis DNA was hybridized with N. gonorrhoeae DNA,

Chromatographic Cycle	^{32}P N.Gon unbound	^{32}P N.Gon EDTA eluted
1	66%	34%
2	74%	26%
3	87%	13%
4	92%	8%
5	99%	1%

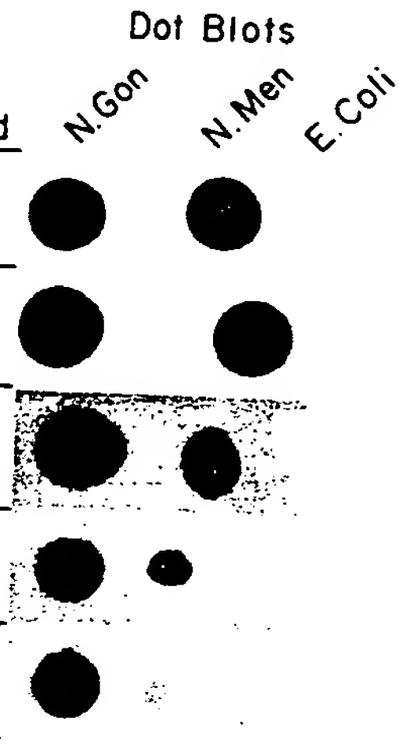


Figure 2. Purification of ^{32}P-labeled N. gonorrhoeae DNA by subtractive hybridization with biotinylated N. meningitidis DNA. After each of the five rounds of solution hybridization the samples were adjusted to 1 M NaCl, Avidin-DN added to a final concentration of 0.5 mg/ml, the samples incubated at 55°C for 10 minutes and then chromatographed over a 0.4 ml bed of cupric-iminodiacetic acid agarose. The distribution of the ^{32}P-labeled N. gonorrhoeae DNA in the non-hybridized (unbound) and hybridized (EDTA-eluted) fractions after each cycle is tabulated. The specificity of the unbound DNA fraction at each cycle against N. gonorrhoeae (N. gon), N. Meningitidis (N. men) and E. coli DNA is illustrated by the dot-blot hybridization reactions on the right.

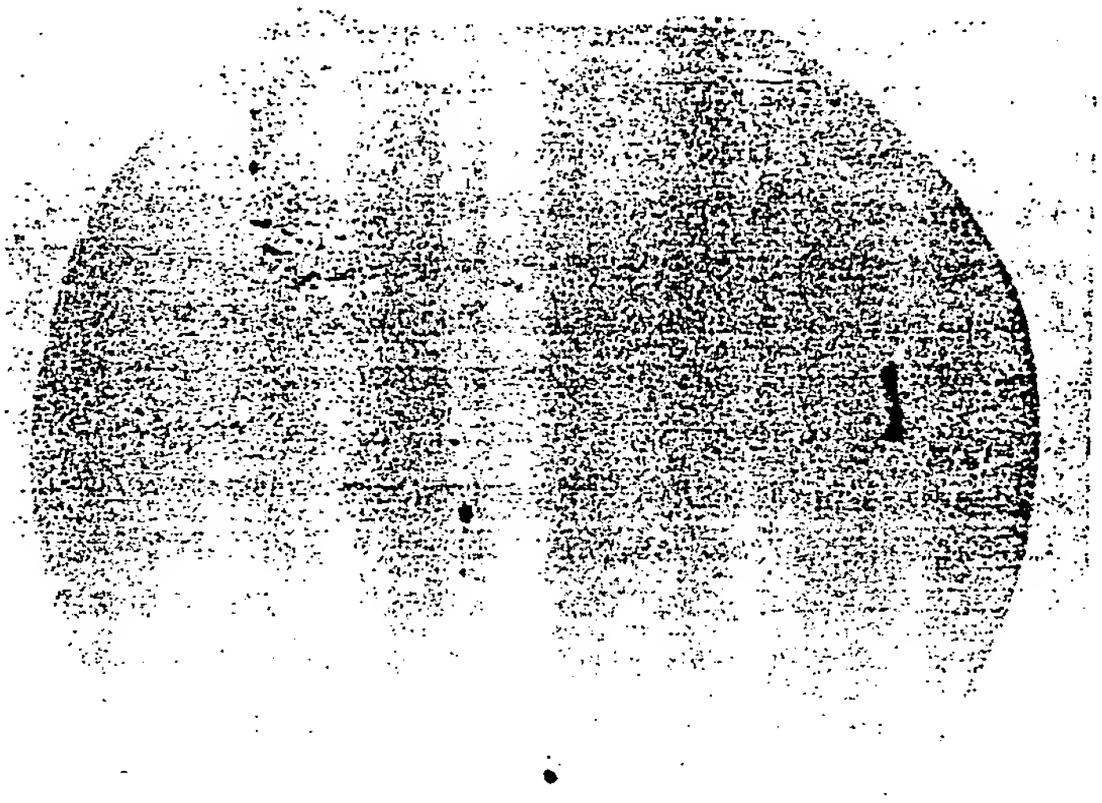


Figure 3. Hybridization to a grid of 100 colonies from a shot-gun library of Sau3AI digested N. gonorrhoeae DNA, cloned into pUC-13 and transformed into E. coli strain JM103. The ^{32}P-labeled probe was the N. gonorrhoeae DNA subset obtained after five cycles of subtractive hybridization and copper-chelate chromatography. Only five of the 100 colonies show positive hybridization.

incubated with avidin, and then chromatographed over a copper-chelate column as described in Methods and Materials. In cases where the target DNA was not radiolabeled, an aliquot of the DNA in the flow-through fraction was ^{32}P-labeled using polynucleotide kinase and then used to monitor enrichment by dot-blot hybridization to the two bacterial DNAs. The remaining DNA was hybridized again to excess meningitidis DNA. The hybridization-fractionation cycle was repeated 3 or 4 additional times or until a strong dot-blot hybridization signal was obtained only from the N. gonorrhoeae DNA.

The results of a subtractive enrichment experiment, using N. gonorrhoeae DNA prelabeled with ^{32}P, is shown in Figure 2. With each additional hybridization-chromatography cycle the percentage of the ^{32}P-N. gonorrhoeae DNA that was retained on the copper-chelate resin decreased; after the fifth round of hybridization only 1% of the input DNA was bound. The remaining 99% of the DNA in the flow-through fraction, representing 5% of the original N. gonorrhoeae DNA, also give virtually no signal when hybridized to N. meningitidis DNA. This preenriched, ^{32}P-labeled, DNA was then used to screen

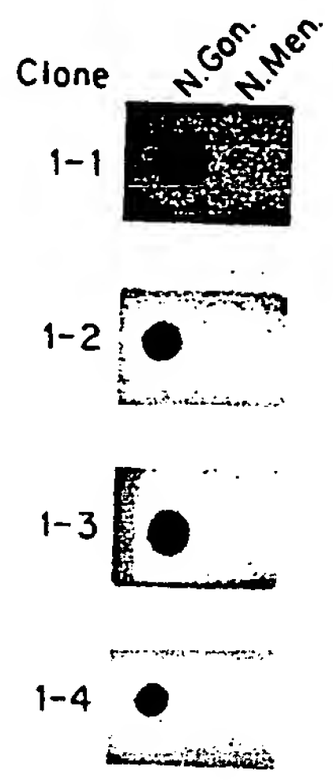


Figure 4. Plasmid clones identified by the ^{32}P-labeled DNA subset obtained after five cycles of subtractive hybridization and copper-chelate chromatography are specific for N. gonorrhoeae by dot-blot hybridization. Clones 1-1, 1-2, 1-3 and 1-4 had DNA inserts of 1.2, 0.5, 0.3 and 1.0 kb, respectively.

a library of Sau3A-digested N. gonorrhoeae DNA that had been cloned directly into the Bam HI site of pUC-13. Nitrocellulose filters, each containing 100 transformants, were prepared and subjected to colony hybridization as described in Methods. On average only 5% of the colonies in any grid gave a positive hybridization signal (Figure 3). Screening the shot-gun library with preenriched DNA as a probe was the most efficient way to identify multiple N. gonorrhoeae specific clones. Direct cloning of the enriched DNA subpopulation after reannealing gave less reproducible results, mainly due to variations in the ligation and transformation efficiences.

Plasmid DNA preparations were prepared from a total of 8 positive clones and the DNA insert sizes determined by gel electrophoresis; the size distribution, from 0.2 to 1.5 kb with an average of 1.1 kb, was only slightly smaller than that seen for the total genomic DNA after Sau3A digestion. These plasmid DNAs were ^{32}P-labeled by nick-translation and hybridized to dots of N. gonorrhoeae and N. meningitidis DNA. Seven of the 8 clones tested were found to be specific for N. gonorrhoeae. Typical hybridization results are exemplified by the four clones illustrated in Figure 4. In contrast, when 22 clones were picked randomly from the library and screened in a similar fashion only 1 of the plasmids hybridized uniquely to N. gonorrhoeae DNA.

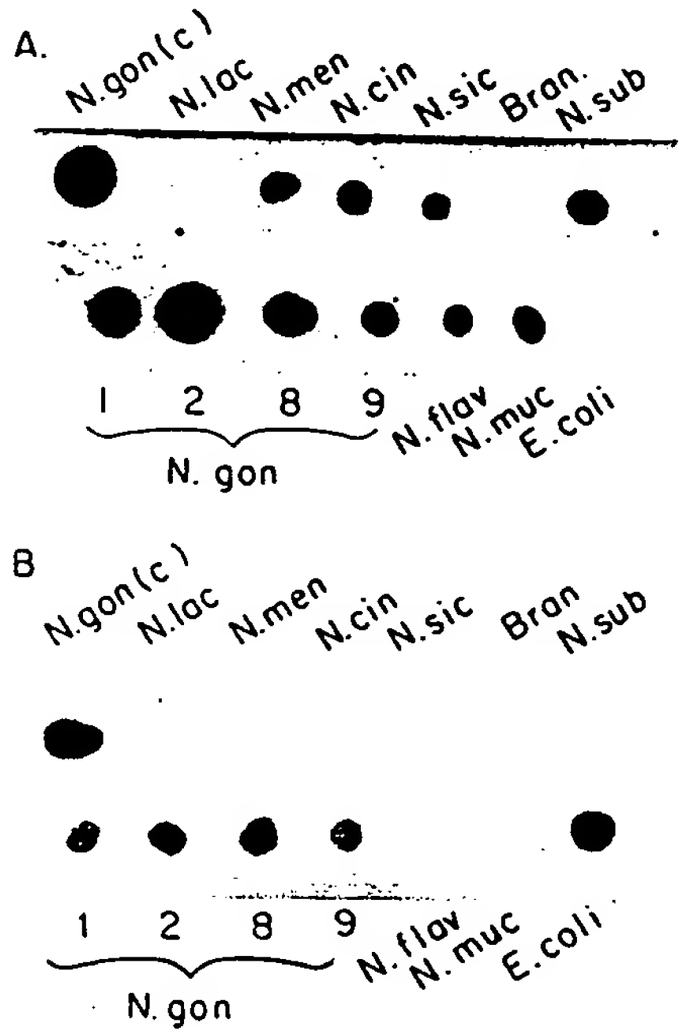


Figure 5. N. gonorrhoeae clones, detected by preenriched DNA probes, specifically hybridize to N. gonorrhoeae DNA. Panel A. N. gonorrhoeae genomic DNA, ^{32}P-labeled by nick-translation, hybridizes both to N. gonorrhoeae strains (the origin strain [N. gon (c)] and clinical isolates 1,2,8 and 9) as well to DNA of other Neisseria strains; N. lacamica (N. lac), N. meningitidis (N. men), N. cinerea (N. cin), N. sicca (N. Sic), N. subflava (N. sub), N. flavescens (N. flav) and N. mucosa (N. muc). No hybridization is observed with Escherichia coli (E. coli) or Branhamella catarrhlis (Bran) DNAs. Panel B. ^{32}P-labeled Clone 1-3 DNA, containing a 0.3 kb fragment of N. gonorrhoeae DNA, hybridized to all N. gonorrhoeae isolates but not to the DNA of other Neisseria strains.

Several of the clones identified with the preenriched DNA probe set were also tested for cross-hybridization with 7 other Neisseria species and for their ability to identify clinical isolates of N. gonorrhoeae. As shown in Figure 5A, total genomic DNA from N. gonorrhoeae hybridized strongly to all Neisseria species tested except N. lactamica. No hybridization was observed with E. coli or Branhamella catarrhlis, two non-Neisseria DNA controls. In contrast, clone 1-3 DNA hybridized significantly only to the 5 N. gonorrhoeae isolates (Figure 5B). Similar results were obtained with other clones. The signal seen with E. coli DNA in Figure 5B is an artifact, and reflects traces of contaminating E. coli sequences in the plasmid preparation which become

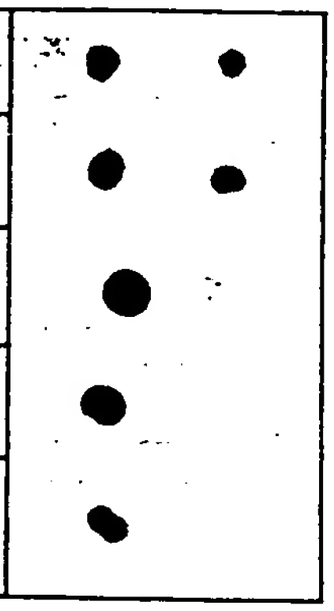
Chromatographic Cycle	^{32}P cDNA Unbound	^{32}P cDNA EDTA eluted	Dot Blots: Induced RNA / Uninduced RNA
0	—	—	
1	45 %	55 %	
2	52 %	48 %	
3	72 %	28 %	
4	93 %	7 %	

Figure 6. Purification of cDNAs specific to DMSO-induced MEL-745 cells. ^{32}P-labeled cDNAs prepared from poly(A)$^+$ RNA expressed 72 hours after treatment with DMSO, were hybridized with an excess of poly(A)$^+$ RNA from induced cells a total of 4 times. After each hybridization reaction the sample was incubated with Avidin-DN and chromatographed over a copper-chelate resin (see Methods). The distribution of ^{32}P-labeled cDNA in the non-hybridized (unbound) and hybridized (EDTA-eluted) fractions after each cycle is tabulated and the hybridization signal of each unbound cDNA fraction against induced and uninduced poly(A)$^+$ RNA is shown.

radiolabeled during nick-translation (data not shown). Such artifactual signals can be eliminated by subcloning the insert into SP-6 or T-phage transcription vectors and using the insert-specific transcripts as hybridization probes.

Purification of cDNAs Complementary to mRNAs Overexpressed in DMSO-induced Friend Cells

Murine erythropoietic precursor cells transformed by Friend Virus, termed Friend cells, morphologically resemble normal proerythroblasts. When treated in culture with dimethylsulfoxide (DMSO) they differentiate along the erythroid pathway. During these changes there is an accumulation of α and β-globin mRNA and hemoglobin as well as other products characteristic of terminal erythropoiesis (26). To test the fractionation method further we have used poly(A)$^+$ RNA from an uninduced Friend cell (MEL-745), chemically labeled with photobiotin, to purify a cDNA subpopulation which hybridizes only to poly(A)$^+$ RNA prepared from MEL 745 cells 72 hours after DMSO-induction. Biotinylated RNA and ^{32}P-labeled first-strand cDNA were prepared, hybridized,

and chromatographed over the copper-chelate resin as described in Methods. As shown in Figure 6, the cDNA hybridizes extensively to both induced and uninduced RNA prior to subtractive enrichment. In contrast, after four cycles of hybridization and chromatography the ^{32}P-cDNA hybridized exclusively with RNA from induced cells. This cDNA subpopulation represents 7 percent of the original cDNA pool. Affara and Daubas (6) had previously shown that 70-75% of the induction specific cDNA, separated by HAP chromatography, is complementary to α and β-globin mRNA. The cDNA subpopulation selected via the copper-chelate agarose also hybridized extensively with α and β-globin genomic DNA clones (data not shown). The cloning and further characterization of this cDNA subpopulation will be reported elsewhere. It is clear, however, that the biotin-avidin-copper-chelate separation method described here can be used as an efficient alternative to HAP-chromatography for selective cDNA purification.

DISCUSSION

We have described a versatile method for the purification of selected nucleic acid sequences and demonstrated its utility in preparing N. gonorrhoeae specific genomic DNA clones and a differentiation specific cDNA subpopulation. Although the experiments reported here required four or five cycles of subtractive hybridization and chromatography, two cycles are usually sufficient for enrichment when higher concentrations of driver probes are used. Furthermore, when selecting target sequences of relatively low abundance, it is recommended that the biotinylated probe pool be incubated with an excess of avidin, then bound to and eluted from the copper-chelate agarose prior to use. This ensues that all probe molecules added to hybridization reactions can be quantitatively recovered during subsequent chelate chromatography, thus minimizing the potential of contaminating target sequences with non-biotinylated probes. Since biotinylated probes eluted from the copper-chelate column with EDTA will be complexed with avidin, the protein must be removed, by proteinase K-pronase digestion and phenol extraction, in order to maintain optimum hybridization kinetics.

This technique can be applied to a wide variety of separation problems where no information concerning specific target DNA sequences, their transcripts or their gene products is available. For example, in species such as bacteria, where the genome contains few, if any, highly repetitive sequence elements that can lower selective enrichment by generating interstrand networks, one could readily isolate large sets of organism specific sequences.

Enrichment by subtractive hybridization potentially provides a rapid means of preparing extensive probe sets which could be useful in the clinical diagnosis of specific pathogens. Conversely, one could analyze the sequence subsets retained on copper-chelate agarose to identify conserved sequences that are common to an entire family or genera of organisms. The subtraction hybridization method also could be used to identify and clone DNA segments of unknown sequence which have been deleted during strain evolution, mutagenesis, or as a direct or indirect consequence of pathologic disease (27). In screening the genomes of higher organisms that possess numerous repetitive elements, unique sequence subsets could be prepared from somatic cell hybrids, clone banks, or individual chromosomes by subtractive hybridization with appropriate repetitive sequence probes, e.g., the Alu, Kpn or centromer family repeats. These and other applications of this fractionation method are currently being evaluated.

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*To whom correspondence should be addressed

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